Toxicity of ammonia and nitrite to silver perch (*Bidyanus bidyanus*)

A thesis submitted in fulfilment of the requirements

for the degree of

Master of Applied Science

University of Tasmania, Launceston

by

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December 1998

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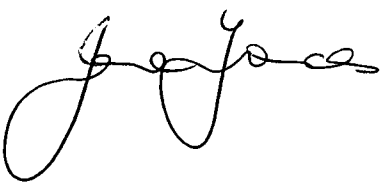

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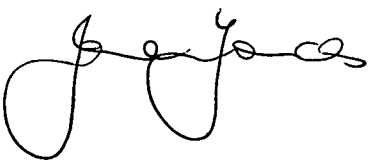

4 December 1998

Abstract

Acutely toxic concentrations (LC_{50}) of ammonia and nitrite were estimated for juvenile silver perch (*Bidyanus bidyanus*) during 96h experiments. Additional experiments were conducted for at least 25 days to determine the growth-limiting concentrations of ammonia and nitrite for juvenile silver perch. Furthermore, the effects of exposure to metabolic and reagent ammonia were compared. Gill histopathology was examined in each experiment to determine if changes in gill structure correlated with exposure to toxicant, and whether gill histopathology was a useful indicator of sub-acute intoxication for this species.

The acutely toxic concentration (LC_{50}) of un-ionised ammonia (UAN) was defined as the concentration at which silver perch lost the ability to orientate. At this point, fish were removed from the experiment, as preliminary research had shown that prolonged exposure beyond this point always led to death. The LC_{50} was estimated to be 1.2 mg L^{-1} UAN. The acutely toxic concentration (LC_{50}) of nitrite was estimated to be 160 mg L^{-1} NO_2-N.

Following a 39 day exposure, growth (wet weight gain) of juvenile silver perch was reduced at concentrations of ammonia above 0.36 mg L^{-1} UAN. There was no significant difference in wet weight gain of control fish or those exposed to 0.02 mg L^{-1} metabolic ammonia. Growth of silver perch exposed to nitrite at concentrations above 1.43 mg L^{-1} NO_2-N for 25 days was reduced.

Following acute exposure to ammonia, there was a significant increase in the percentage of filaments affected by epithelial lifting, but no other significant change in gill histopathology was observed. Acute exposure to nitrite resulted in no significant changes in gill histopathology, although macroscopic observation indicated browning and discolouration of gill tissue at concentrations above 130 mg L^{-1} NO_2-N.

After 39 days' exposure to ammonia, there was an increase in the occurrence of epithelial lifting, while no other index of gill histopathology was significantly different. Exposure to metabolic ammonia also increased the percentage of filaments affected by epithelial lifting. Following 25 days' exposure to nitrite, there was a significant difference in the percentage of gill filaments affected by epithelial lifting and hypertrophy.

These results indicate critical concentrations of ammonia and nitrite which will assist in the management of nitrogenous wastes in pond or tank culture of silver perch. Understanding of these concentrations may increase production and reduce the incidence of critical water quality conditions.

Acknowledgement

I thank my co-supervisor, Dr Geoff Allan, for advice and for providing me with the opportunity to undertake this research, and I am especially grateful to my supervisor, Dr Barbara Nowak, for introducing me to new approaches and for her enthusiasm and encouragement at all times.

I acknowledge the support of the staff of NSW Fisheries Port Stephens Research Centre. In particular I am grateful for the assistance of Dr Wayne O'Connor, Ms Jo Pickles, Mr Ian Smith, Ms Rebecca Warner-Smith and Mr Mark Booth. Provision of fish and advice from Dr Stuart Rowland at NSW Fisheries Grafton Research Centre is gratefully acknowledged. The efficient assistance of NSW Fisheries librarians, Ms Caroline Bland and Ms Kathy Bown, was invaluable.

I also thank Mr Ralph de Ville for provision of laboratory space at Hunter Institute of TAFE, and Ms Debbie Pepperall for assistance in the preparation of histological material.

Finally, I thank my friends and family for their support, and especially my mother, who has been a life-long inspiration.

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CHAPTER 1

General Introduction

General Biology of Silver Perch

Silver perch *Bidyanus bidyanus* (Mitchell) is a member of the Family Teraponidae. Teraponids are found widely distributed throughout the Indo-Pacific, and include both salt- and freshwater representatives. Approximately 30 species are found in Australia, largely in tropical northern Australia. Two species are found in temperate Australia. The spangled perch *Leiopotherapon unicolor* is restricted to the Darling River and its tributaries. Silver perch have a more extensive range, and are found in the Murray/Darling Rivers catchment and associated tributaries in Victoria, New South Wales, South Australia and into southern Queensland. Wild-caught silver perch supported a commercial fishing industry at the turn of the century. Their range and abundance has been greatly restricted in recent years (Cadwallader and Backhouse, 1983), largely due to the construction of dams and weirs and the resultant changes to its habitat. Silver perch are currently listed as vulnerable (Schiller et al., 1997).

Life History of Silver Perch

Female silver perch mature at 2-3 years, at which time they usually weigh between 1-2 kg. Male silver perch mature in their second year and weigh approximately 1 kg. In the wild, spawning usually occurs between November and March during summer

flooding, and requires water temperatures of 23°C and a minimum rise in water level of approximately 150 mm (Lake, 1967). Female silver perch can produce up to 400 000 eggs per season. All eggs are usually shed during a single spawning event. Hatching takes place approximately 30 h after fertilisation at temperatures between 23-30°C. Silver perch larvae possess a prominent oil globule and yolk sac and do not require feeding until Day 5-6 post-hatching (Thurstan and Rowland, 1995). First feed silver perch have a relatively small gape (4 mm) and will feed on small rotifers, chironomid larvae and small crustaceans (Thurstan, 1991). After hatching, larvae develop into fry and finally metamorphose to become juveniles or fingerlings. Fry usually metamorphose within 18 days, at which stage the juveniles are approximately 11 mm. Growth rates are temperature-dependent and extremely variable, with an average growth of approximately 100 mm per year during the first two years in the wild (Cadwallader and Backhouse, 1983).

Aquaculture of Silver Perch

Established hatchery techniques for the production of silver perch rely on hormone-induced spawning, using Human Chorionic Gonadotrophin (Rowland, 1984, 1986). Silver perch are omnivorous, tolerate crowding, grow rapidly and readily accept artificial feeds (Rowland and Barlow, 1991). These features, in addition to the possession of firm, white flesh and a high meat recovery rate, have made silver perch an attractive candidate for semi-intensive aquaculture production in earthen ponds (Rowland et al., 1995).

Recent successes in the production of silver perch indicate its aquaculture potential (Rowland, 1995a; Rowland et al., 1995). Production rates of 10 t ha^{-1} yr^{-1} have been achieved in static, aerated earthen ponds (Rowland et al., 1995), however, high concentrations of un-ionised ammonia have been linked to slow growth and disease in silver perch (Rowland, 1995a; Rowland et al., 1995). Larmoyeux and Piper (1973) also observed reduced growth in farm-reared rainbow trout *Oncorhynchus mykiss* at elevated ammonia concentrations. In these field-based studies, concomitant high concentrations of nitrite, though not reported, appear likely, and may have contributed to observed growth suppression.

The purging of live, market-size (450-500 g) fish is a quality control measure which has been adopted by the silver perch industry to allow for the elimination of "off-flavours" prior to marketing. Off-flavour compounds are generally produced by blue-green algae and actinomycetes which can proliferate during fish production in earthen ponds (Sevsin-Reyssac and Pletikosic, 1990) and can accumulate in silver perch, especially in fatty deposits (Rowland, 1995c). Holding harvested, live, unfed fish in clean water for five to fifteen days allows for the purging of off-flavours, but crowding of large fish may result in elevated levels of both ammonia and nitrite.

Ammonia

Ammonia is one of the most important water quality variables in freshwater aquaculture. Ammonia is the major end product of protein catabolism in fish (Campbell, 1973) and can accumulate to toxic levels in intensive culture systems or where water is reused. Ammonia accounts for 80% of the total nitrogen excreted by

freshwater fish (Smith, 1929) and is highly toxic to fish (Tomasso, 1994). The degree of sensitivity to ammonia toxicity is species-specific (EIFAC, 1970), and is further influenced by life stage (Bader and Grizzle, 1992: Burkhalter and Kaya, 1977; Thurston et al., 1986). In intensive culture, ammonia can accumulate rapidly and can compromise the health, growth and survival of the cultured species. After dissolved oxygen, nitrogenous compounds are the most important water quality parameter limiting aquaculture production (Colt and Armstrong, 1981). Knowledge of the water quality requirements of the target species is essential to promote production.

In solution, ammonia exists in two forms in equilibrium, represented by the equation:

$$NH_3 + H_2O \Leftrightarrow NH_4^+ + OH^-$$

The un-ionised form is considered the most toxic (Downing and Merkens, 1955; Wurhmann and Woker, 1948). It is a highly soluble gas which readily crosses biological membranes, as it is lipid soluble and uncharged (Fromm and Gillette, 1968).

Throughout the text, the abbreviation UAN refers to un-ionised ammonia nitrogen, or NH_3-N, while TAN refers to total ammonia nitrogen, or $NH_4^+ + NH_3$, a convention similar to that used by several other authors (Colt and Armstrong, 1981; Bader and Grizzle, 1992).

Ammonia is a volatile weak base (Cameron and Heisler, 1983) and the proportion of UAN present for a given concentration of total ammonia TAN is largely influenced

by pH (Wuhrmann and Woker, 1948; Emerson et al., 1975). As pH rises, so too does the proportion of un-ionised ammonia (EIFAC, 1970; Colt and Armstrong, 1981; Randall and Wright, 1987; Schaperclaus et al, 1992). Burrows (1964) stated that for every increase of one pH unit there is a corresponding 10 fold increase in UAN. However, there are some discrepancies in the literature regarding the influence of pH on ammonia toxicity (see reviews of Meade, 1985; Russo and Thurston, 1991; Tomasso, 1994). Sheehan and Lewis (1986) observed an apparent increase in the toxicity of UAN to age-0 channel catfish *Ictalurus punctatus* at low pH and attributed this to isotonic dehydration. Further, Thurston et al. (1981a) found the increase in ammonia toxicity for rainbow trout *Oncorhynchus mykiss* at increasing pH could not solely be attributed to an increased proportion of UAN present.

In general, it is believed that diffusion of ammonia across the gills is governed by concentration gradients, where UAN diffuses out of the fish if the external concentration is lower than that in the fish (Wise et al., 1989; Weirich et al., 1993). Given our understanding of the ammonia dissociation equilibrium, the percentage of ammonia present as UAN is influenced by the internal pH of the fish. Likewise, the same can be said for conditions in the water, however variations in pH in the immediate vicinity of the gill membrane (due to carbon dioxide efflux) create local conditions which may be quite different from ambient and may strongly affect the diffusion gradient (Randall, 1991). Furthermore, there is evidence of the presence of an active transport mechanism in some species (Evans and Cameron, 1986), which further complicates our ability to predict the nature of the relationship between ammonia toxicity and pH.

Temperature also affects the position of the equilibrium, but to a lesser extent than pH. A rise in temperature of seawater from 10°C to 20°C approximately doubles the percentage of ammonia present as UAN for pH 7.5-8.5 (Bower and Bidwell, 1978).

Nitrite

Ammonia and nitrite are the major metabolic end-products of fish (Colt and Tchobanoglous, 1976) and both are toxic to fish (Tomasso, 1994). Nitrite is the intermediate product in the oxidation of ammonia to nitrate (Colt and Armstrong, 1981), and can accumulate rapidly in intensive culture systems, particularly in systems using recirculated water (Perrone and Meade, 1977; Holt and Arnold, 1983) or in systems where fish are crowded.

In solution, nitrite exists as nitrite ion (NO_2^-) and un-ionised nitrous acid (HNO_2) in equilibrium, according to the equation:

$$HNO_2 \Leftrightarrow NO_2^- + H^+$$

The position of the equilibrium is greatly influenced by pH (Russo et al., 1981). With rising pH the fraction of nitrous acid present decreases. The proportion of each is influenced to a lesser degree by temperature (Colt and Tchobanoglous, 1976). As is the case with ammonia, the un-ionised form is lipophilic and diffuses readily across cell membranes. However, at pH above 4, more than 90% of nitrite exists in the ionised form, and in the pH range commonly encountered in aquaculture, the proportion of nitrite in the ionised form comprises more than 99.9% (Tomasso,

1994). It has been suggested that the nitrite ion is actively transported across the epithelial cells via the active transport mechanism which transports chloride ions (Perrone and Meade, 1977; Margiocco et al., 1983). However, several authors have suggested that the toxicity of nitrite is in fact brought about through the action of nitrous acid.

The oxidation of ammonia to nitrite and ultimately nitrate is known as nitrification, and is facilitated in a step-wise process by two groups of bacteria, *Nitrobacter* and *Nitrosomonas* (Stanier et al., 1970), according to the following equations:

1. $2NH_4^+ + 3O_2 \xrightarrow{\text{Nitrosomonas}} 2NO_2^- + 4H^+ + 2H_2O$

2. $4NO_2^- + 2O_2 \xrightarrow{\text{Nitrobacter}} 4NO_3^-$

Both groups have relatively slow growth rates, and do not respond quickly to sudden increases in nitrogenous waste loads (Colt and Tchobanoglous, 1976). Therefore the process is relatively efficient, in terms of ammonia conversion, in systems where water flow is moderate and ammonia load is relatively low. Concentrations of nitrite can accumulate to toxic levels where water is re-used and where biological load is high (Larmoyeux and Piper, 1973; Westin, 1974).

Published literature reveals some conjecture regarding the primary mechanism of nitrite toxicity, with some authors noting various organs are subject to detrimental effects following nitrite exposure, suggesting that the toxic action of nitrite on one or more vital organs may be fundamentally responsible for nitrite toxicity. Arillo et al.

(1984) found ultrastructural changes to the liver of rainbow trout *Oncorhynchus mykiss* exposed to nitrite and concluded that liver hypoxia is the root of the nitrite acute toxicity mechanism. Mensi (1982) also suggested that nitrite is hepatotoxic, reporting lysosomal damage to rainbow trout *Oncorhynchus mykiss* exposed to nitrite. Mazik et al. (1991) suggested that nitrite exposure elicits primary and secondary stress responses in striped bass *Morone saxatilis* through elevated plasma cortisol and glucose. Such changes in plasma electrolyte balance may result in osmoregulatory dysfunction. Bowser et al. (1989) found nitrite exposed Atlantic salmon *Salmo salar* exhibited elevated alamine aminotransferase (ATPase) in blood plasma, which is indicative of generalised cell injury. Further, Langdon (1987) confirmed ATPase participation in the exchange of Cl^- and HCO_3^- in Australian bass *Macquaria novemaculata* and golden perch *Macquaria ambigua.* Margiocco et al. (1983) found that rainbow trout *Oncorhynchus mykiss* exposed to nitrite had elevated concentrations of nitrite in various organs, including liver, gills and brain, and concomitant scant correlation between physiological state and methemoglobin concentration in the blood. Michael et al. (1987) observed gill damage in juvenile African catfish *Clarias lazera* exposed to 2.8 mg L^{-1} NO_2-N for 6 months, while kidneys of exposed fish were not histologically different from controls. It appears possible that more than one organ is involved in the mechanics of nitrite toxicity in fish. Furthermore, the primary mechanism of nitrite toxicity may be species-specific.

A variety of conditions have been associated with exposure to nitrite in freshwater fish. These include methemoglobinaemia, or brown blood disease (Brown and McLeay, 1975), ultrastructural changes to liver (Arillo et al., 1984), lysosomal damage (Mensi, 1982), osmoregulatory dysfunction (Mazik et al., 1991), and

generalised cell injury (Bowser et al., 1989). Many studies have included histological examination of gill tissue as an indicator of sub-lethal effects on fish health.

Physiology

Acute and/or chronic bioassays have been used to establish water quality criteria (EIFAC,1970; Sprague, 1970, 1971; Heath, 1987). Acute bioassays classically establish LC_{50}s, which is defined as the concentration which is lethal to 50% of the population (Sprague, 1969) in a given time period (often 96h - see Tables 2 and 7 of this thesis). Chronic bioassays use a variety of physiological characteristics as a measure of the impact of toxicants, including fecundity (Thurston et al., 1984, 1986), the nature and action of corticosteroids (Jeney et al., 1992a), blood chemistry (Swift, 1981; Almendras, 1987; Bowser et al., 1989; Wlasow and Dabrowska, 1990; Jeney et al., 1992b; Oppenborn and Goudie, 1993; Schoore et al., 1995), acid/base disturbance (Wright and Wood, 1985), Na^+ balance (Cameron and Heisler, 1983; Twitchen and Eddy, 1994) disease resistance (Schreckenbach et al., 1975; Burkhalter and Kaya, 1977; Plumb, 1984; Hanson and Grizzle, 1985; Hurvitz et al., 1997) and growth (Smith and Piper, 1975; Colt et al., 1981; Colt and Tchobanoglous, 1978; Soderberg et al., 1983; Bader and Grizzle, 1992; Kamstra et al., 1996; Rasmussen and Korsgaard, 1996). In the present study, the concentration of ammonia and nitrite beyond which growth is impaired by 5% was determined. The measurement of growth is rapid, accurate and non-intrusive. Furthermore, determining concentrations of toxicants which reduce growth is of obvious benefit to the aquaculture industry in terms of improving profitability.

Histology

The gills of teleost fish are a multifunctional organ. Excretion, osmoregulation and respiration take place across the gills. Fish gills have a large surface area covered by a thin layer of epithelial cells, well supplied with blood to facilitate gas exchange (Hughes, 1984). As a result, gills are often most affected by exposure to toxicant and histological examination of gill tissue may reveal toxicant-induced structural changes (Mallatt, 1985).

The structure of the fish gill consists of 2 sets of 4 gill arches, each of which bear 2 rows of filaments. These filaments are arranged alternatively and at an angle from each other. They bear the two rows of lamellae, which are fine structures composed of blood spaces divided by pillar cells and enclosed in a thin layer of epithelial cells (Eller, 1975; Amin et al., 1985).

The delicate structure of the gill makes it vulnerable to damage by any irritant or toxicant which may be dissolved or suspended in water. The lamellae are highly vascularised with a large surface area and are the primary site of gas exchange (Hughes, 1984). Histology is a commonly used method of examining the sub-lethal effects of toxicants on fish. Sublethal exposure to irritants frequently caused swelling or hypertrophy of lamellar epithelial cells and epithelial lifting (Mallatt, 1985). Histopathological responses of gills to chronic exposure to irritants include lamellar hyperplasia and lamellar fusion (Roberts, 1989) and ultimately lamellar telangiectasis, or aneurysm (Mallatt, 1985).

In work with channel catfish *Ictalurus punctatus*, Soderberg et al. (1984) commonly found gill lesions in fish exposed to average UAN levels fluctuating between 29 and 67 ug L^{-1}. However, Mitchell and Cech (1983) found that histological evidence failed to confirm ammonia as the causative agent of gill hyperplasia in the same species and postulated that residual chlorine compounds may be a confounding factor. On closer examination of results from published studies, it appears possible that the source of ammonia (whether metabolic or reagent) may influence the histopathological changes observed as a result of toxic insult (Table 1). Results summarised in Table 1 suggest that exposure to metabolic ammonia gives rise to histopathological changes to gill tissue, while exposure to reagent ammonia results in neurological dysfunction and brain lesions. This difference in response to ammonia from different sources may result from the action of, or synergism with, other waste products of metabolic origin, such as urea and uric acid, creatine and creatinine, and amine and amine oxide derivatives (Brockway, 1950; Daoust and Ferguson, 1984; Meade, 1985), which are not present in reagent ammonia solutions. One aim of the present study was to compare histopathological changes to juvenile silver perch exposed to ammonia sourced from reagent with that sourced from metabolic processes.

Histopathology of fish gills has commonly been undertaken to determine sub-lethal effects of toxicants (Leino and M^{c}Cormick, 1984; Nowak, 1992; Kirk and Lewis, 1993) but qualitative analysis makes comparisons between treatments more difficult and perhaps more arbitrary. Histopathological changes in the present study were quantified using proportional morphometry, as described in General Materials and Methods. Quantification of histopathological data allows for statistical analysis and

TABLE 1 Published ammonia toxicity studies - comparative histopathological results

Species	UAN (mg L^{-1})	Duration	Result	Source of ammonia	Reference
Rainbow trout *Oncorhynchus mykiss*	0.8	4 months	Thickening of epithelium covering gill lamellar	Metabolic	Larmoyeux & Piper (1973)
" "	0.003 - 0.01	3 months	Epithelial hypertrophy → tissue necrosis	Metabolic	Peters et al. (1984)
" "	0.5	1 year	Histopathological changes to gill tissue	Metabolic	Smith & Piper (1975)
" "	0.3 - 1.3	120 days	Gills lesions, especially oedema and aneurysms	Metabolic	Soderberg (1985)
Chinook salmon *Oncorhynchus tshawytscha*	0.006	6 weeks	Gill hyperplasia	Metabolic	Burrows (1964)
Channel catfish *Ictalurus punctatus*	0.02 - 0.067		Gill lesions	Metabolic	Soderberg et al. (1984)
Rainbow trout	0.2 & 0.4	90 days	Neuro dysfunction, no gill lesions	Reagent	Daoust & Ferguson (1984)
Oncorhynchus mykiss	0.03	16 weeks	15% reduction in growth	Reagent	Klontz et al. (1985)
" "	0.03	2 weeks	Gill lamellar hypertrophy	Reagent	" "
" "	0.53 ± 0.18	12 weeks	No gill tissue damage	Reagent	Mitchell & Cech (1983)
" "	0.53 ± 0.18	12 weeks	Hyperplasia	Reagent + Chloride	" "
" "	0.25 - 0.3	36 days	Thickening of lamellar epithelium but not gill hyperplasia	Reagent	Smart (1976)
Fathead minnows *Pimephales promelas*	0.21	1 year	Brain lesions	Reagent	Smith (1984)
" "	0.21 - 0.96	1 year	Brain lesions, no other histopathology	Reagent	Thurston et al. (1986)

In general: Metabolic ammonia ⇒ histopathological changes, gill hypertrophy, gill lesions
Reagent ammonia ⇒ brain lesions, neurological disfunction

for comparison with significant physiological responses traditionally measured in toxicity studies, such as growth (Johnson and Bergman, 1984).

Objectives of the present study

The aims of this study were:

i) to determine the concentration of ammonia and nitrite which juvenile silver perch can tolerate;

ii) to determine the growth-limiting concentrations of ammonia and nitrite to juvenile silver perch;

iii) to investigate if structural changes in gill tissues are induced by exposure to ammonia or nitrite; and

iv) to determine if juvenile silver perch exhibit any difference in growth or gill histopathology when exposed to either metabolic or reagent ammonia.

These objectives were investigated during two acute (96 h) experiments and two chronic (25-39 day) growth-limiting experiments.

CHAPTER 2

General Materials and Methods

Fish

All fish were produced under similar conditions at NSW Fisheries' Grafton Research Centre using hatchery techniques and extensive pond rearing described by Rowland (1984; 1995a). Fish were transported to and held at NSW Fisheries' Port Stephens Research Centre in either concrete or fibreglass tanks for at least 2 weeks until required. During this time, fish were fed a 35% protein silver perch ration (SP35; Allan and Rowland, 1992) *ad libitum* twice daily.

For all experiments, fish were graded (to minimise size variation within each experiment) using a manual fish grader prior to stocking. In the case of 96 h experiments, a subsample of fish ($n \geq 40$) were taken at random, anaesthetised and weighed to indicate mean weight and range of fish used in that experiment.

For all experiments, fish were stocked into experimental aquaria and acclimated for at least 5 days in the temperature and photoperiod controlled laboratory in which experiments were conducted prior to the commencement of the experiment. All experiments were conducted in replicated aquaria. All fish were fed twice daily *ad libitum* during acclimation, at the same time of day as during the growth-limiting experiments. Fish were not fed during acute experiments.

During the growth-limiting experiments, fish were fed twice daily (40% am, 60% pm) with feed rates adjusted daily according to a scoring system based on the following criteria:

Score	0	no feed pellets left	increase daily ration by 0.3 g
	1	1-2 feed pellets left	leave daily ration the same
	2	3-5 feed pellets left	decrease daily ration by 0.15 g
	3	>5 feed pellets left	decrease daily ration by 0.3 g

Scoring was undertaken approximately 30 minutes post feeding, and any uneaten feed was siphoned from the aquaria at that time. At the end of the experiment, collected uneaten feed from each aquarium was oven dried to determine Food Conversion Ratio (FCR) and Specific Growth Rate (SGR). Faeces was siphoned from aquaria as required (1-2 times weekly).

For growth-limiting experiments, fish were weighed individually at the beginning of each experiment and at termination. Dead fish were removed from aquaria and weighed. Mortalities were replaced with fin-clipped fish of a similar size to maintain stocking density. Feed rates were adjusted accordingly. Initial and final weights of replacement fish were excluded from FCR and SGR calculations.

Experimental Procedures

The 96 h experiments were conducted following limit-testing procedures, in accordance with Animal Care and Ethics guidelines. Threshold concentrations

established in preliminary experiments were used as the highest concentration tested in 96 h experiments. Fish were observed regularly and when the equilibrium of an individual fish was disturbed such that it lost the ability to orientate, it was removed from the aquaria, euthenased and sampled for histological preparation. These fish were not replaced.

The effect of each of ammonia and nitrite on the ability to orientate (96 h experiments) and on short-term growth of silver perch were investigated under continuous-flow bioassay in the present study. Most chronic toxicity studies employ a continuous-flow bioassay technique to determine toxicity, as static systems are inferior for determining toxicology in aquatic systems (Chandler et al., 1974). Flow-through bioassays reduce fluctuations in toxicant dosage attributable to metabolic waste accumulation (Alabaster and Abram, 1965) and reduce the stress to which test animals are subjected.

During the 96 h experiments in the present study, flow was continuous from 1 000 L header tanks, with flow briefly interrupted while header tanks were refilled and dosed with toxicant every 24 hours. One header tank was ascribed to each of the concentrations tested. One reservoir was filled with particle filtered (<10μm), pre-heated (25°-27°C) water, from which the header tanks were filled each day. After 5 days acclimatisation, flow to aquaria was temporarily stopped. The header tanks were dosed with toxicant to achieve the nominal concentrations listed in each experiment. This procedure was repeated 24 hourly throughout the experiment. Flow from header tanks to aquaria was then recommenced and maintained at a flow rate of 200 mL min^{-1}.

Chronic toxicity tests were conducted for at least 25 days under a flow-through regime, where water flow rates were maintained at 203 mL min^{-1}. Peristaltic pumps were used to administer the toxicant to a 500 mL mixing chamber at 3 mL min^{-1}. Pre-heated (24.7-27.2°C) particle filtered (<10 μm) laboratory test water was introduced into the mixing chamber at 200 mL min^{-1} and mixed with incoming toxicant in the chamber. Toxicant-dosed water then flowed via gravity into the experimental aquaria at 203 mL min^{-1}.

For every experiment, three replicates of each test concentration and of the control were established. Test concentrations were assigned to aquaria using a randomisation procedure. Analytical Reagent grade NH_4Cl and $NaNO_2$ were used in the ammonia and nitrite experiments, respectively.

Water Quality

Water quality was monitored fortnightly during the holding period. At no time did ammonia and nitrite concentrations exceed 0.01 mg L^{-1} NH_3-N and 0.02 mg L^{-1} NO_2-N respectively in the holding tanks. Temperature in the holding tanks was maintained at 21-24°C.

During the 96 h experiments, pre-heated water was supplied to the header tanks. Water temperature was maintained using a thermostatically controlled air conditioning system and fans to promote circulation. For the growth-limiting experiments, water temperature was maintained using a 6 kW in-line heater and thermostatically controlled air conditioning.

During nitrite experiments, nitrite-nitrogen (NO_2-N) concentrations were determined daily according to the method described in Major et al. (1972). During ammonia experiments, total ammonia-nitrogen (TAN) was determined daily using the indophenol method described in Dal Pont et al. (1974). Un-ionised ammonia (UAN) concentrations were calculated from TAN concentrations, pH and temperature after the method of Bower and Bidwell (1978). The relevant results of these measurements and the ranges of all water quality variables for each experiment are presented in each chapter of this thesis.

Dissolved oxygen, temperature and pH were measured daily using Yeokal Water Quality Meter Model N° 611 (Yeokal Electronics Pty Ltd, NSW, Australia). This meter was calibrated for dissolved oxygen using saturated freshwater and for pH using NBS phosphate and borate buffers (Chemical Rubber Company, 1971). Temperature was calibrated at the start of the experiment using a Dabros standard thermometer (Dobble Instruments, Sandringham, Victoria, Australia). Alkalinity and hardness were determined at the completion of the experiment using commercial test kits (Aquasonics Pty Ltd, Ingleburn, NSW, Australia). The relevant results of these measurements are presented in each chapter of this thesis.

For the ammonia experiments, pH was further determined using Metrohm Model No 605 pH meter and Metrohm reference and glass electrodes (Metrohm Pty Ltd, Switzerland). During chronic ammonia experiments, NO_2-N was determined for each replicate aquaria once weekly, by the method mentioned previously. In the same way, during chronic nitrite experiments, UAN was also determined weekly.

All colourimetric measurements were determined using a Varian Spectrophotometer Model N° DMS100S (Varian Australia Pty Ltd, Frenchs Forest, NSW, Australia).

Histology

The potential for producing lesions as artefacts of poor fixation was considered in the present study. Anaesthetic was used in the present study prior to excision of gill tissue, as both concussion and decapitation, although widely used (Roberts and Shepherd, 1979), have been shown to induce lamellar telangiectasis in trout *Oncorhynchus mykiss* (Crespo et al., 1988) and salmon *Salmo salar* (Herman and Meade, 1985). In the present study, gills were excised from fish and placed in fixative within one minute to minimise the introduction of post-mortem changes which resemble irritant-induced lesions and may confuse interpretation of results (Walsh and Ribelin, 1975; Speare and Ferguson, 1989). Benzocaine was chosen as anaesthetic, as recommended by Ferguson (1989). Bouin's fixative was chosen for use in this study, as it has been found to minimise the occurrence of artefacts of preservation (Ferguson, 1989), particularly epithelial cell hypertrophy (Speare and Ferguson, 1989). All gill tissue was embedded in the same manner, resulting in similar orientation during sectioning (Johnson and Bergman, 1984). Sections were cut parallel to the surface of the gill filament to promote the clarity of pathological changes, as recommended by Oguri (1995).

Up to 5 fish per aquarium were killed using benzocaine overdose and sampled for histological preparations. The gills were excised within one minute and placed in Bouin's fixative for 24 h, then transferred to 70% alcohol. Tissue was mounted in

wax blocks and sectioned at 4-5μm using Leica Jung Model No RM2025 microtome (Leica Jung Pty Ltd, Germany). Sections were then stained using haematoxylin and eosin. Histological preparations were observed and photographed using x100 and x400 magnification on an Olympus BH-2 microscope and camera (Olympus Optical Company Ltd, Japan).

The quantification of histopathological observations reduces subjectivity (Johnson and Bergman, 1984) and allows for subsequent statistical analysis of results (Hinton et al., 1987). Methods of quantification include morphometric methods, such as the measurement of respiratory distances (Nowak, 1992) using computer image analysis (Munday and Nowak, 1997), quantification of cell types and volumes using sterology (Weibel, 1979; Leino and McCormick, 1984; Mallatt et al., 1995), and proportional morphometry (Speare and Ferguson, 1989). In the present study, histopathological changes are reported based on proportional morphometry of branchial tissue (Speare et al., 1997) which readily allows for integration of histopathological results with quantitative physiological data, such as growth data of the present study.

In the present study, results are presented as percentage of filaments affected to enable quantitative analysis. Specifically, observations included:

i) epithelial lifting index: the percentage of lamellae showing areas where epithelial cells have separated from branchial architecture

ii) hypertrophy index: the percentage of lamellae showing evidence of swelling of epithelial cells

iii) lamellar fusion index: the percentage of lamellae which were fixed to adjacent lamellae

iv) hyperplasia index: the percentage of lamellae showing thickened areas of epithelium

v) aneurysm index: the percentage of lamellae showing areas of telangiectasis

vi) epitheliocystis index: the percentage of lamellae infected by cysts (observed in growth-limiting ammonia experiment only).

For all indices, at least 20 intact, well-oriented filaments per sample were observed. 'Well-oriented' was defined as those filaments where lamellae of equal length bilaterally were presented. Filaments which were not well oriented were not included in evaluations.

Calculations

Specific Growth Rate (SGR) and Food Conversion Ratio (FCR) were calculated according to the equations below:

SGR Specific Growth Rate (% day^{-1}): $\frac{(\ln \text{final wt} - \ln \text{initial wt})}{\text{time (days)}} \times 100$

FCR Food Conversion Ratio: $\frac{\text{Feed fed (dry wt)}}{\text{(final wet wt - initial wet wt)}}$

Statistics

One-factor ANOVA was used to investigate the effect of toxicant concentration on growth. Homogeneity of variance was confirmed using Cochran's Test (Winer et al., 1991) and comparisons amongst means were made using Student Newman Keuls method (Sokal and Rohlf, 1981).

With respect to histopathological changes to gill tissue, morphometric indices are presented as percentage of lamellae observed which were affected by a given condition. Evaluations for all fish sampled within each aquarium (replicate) were pooled and are presented as treatment means and standard errors. Heterogeneous data were subjected to Arcsine transformation prior to analysis using single-factor ANOVA, as above. All histopathological data were transformed according to the equation $x^1=2$ arcsine $(x+[1/2n])^{0.5}$ to allow for data which included zero values (Winer et al., 1991). Comparisons amongst means were made using Student Newman Keuls method (Sokal and Rohlf, 1981), when significant differences were established by ANOVA. The statistical software program Statgraphics (Statistical Graphics Corporation, Maryland, USA) was used for all statistical analyses.

Studies where death is an end point have been criticised by Animal Welfare groups. The 96 h experiments in the present study were conducted using limit testing procedures, which allow for the estimation of 96 h LC_{50}. Fish were observed

regularly and were removed from aquaria when they lost the ability to orientate. Preliminary experiments established concentrations of ammonia or nitrite which resulted in juvenile silver perch losing the ability to orientate. Continued exposure to these concentrations following the onset of an inability to orientate resulted in death within 4 h (J Frances, unpublished data). The 96 h concentration at which 50% of fish lost the ability to orientate (estimated LC_{50}) was determined according to the method of Litchfield and Wilcoxon (1949). Published studies generally use death as an end point and refer to calculated LC_{50}. These published studies are used for the purpose of comparison.

CHAPTER 3

The Acute Toxicity of Ammonia to Silver Perch

Introduction

The influence of ammonia toxicity on survival in freshwater fish has been studied extensively (EIFAC, 1970; Russo and Thurston, 1991; Russo, 1992). Published literature indicates that the concentration of ammonia which is acutely lethal to freshwater fish is species-specific and varies widely (Table 2).

The toxicity of ammonia is influenced by a number of water quality variables, in particular pH, temperature and DO. In general, the position of the ammonia equilibrium is directly influenced by increasing pH, resulting in an increase in the proportion of ammonia present in the un-ionised (toxic) form (Bower and Bidwell, 1978). However, the effect of subtle variations in pH at conditions close to optimal varies between species (Sousa et al., 1974; Tomasso et al., 1980). A direct relationship also exists between increasing temperature and ammonia toxicity, again due to the influence of temperature on the ammonia dissociation equilibrium (EIFAC, 1970). There is evidence in the literature indicating a diminished influence at temperatures approaching optimal (Colt and Tchobanoglous, 1976; Thurston and Russo, 1983). Conversely, there is an indirect relationship between UAN toxicity and DO (Downing and Merkens, 1955), which may be attributable to impairment of the oxygen carrying capacity of the blood caused by exposure to elevated ammonia levels (Brockway, 1950).

TABLE 2

The 96 h LC_{50} of ammonia to freshwater fish

Species	Concentration (mg L^{-1} UAN)	Comment	Reference
Channel catfish *Ictalurus punctatus*	3.8	30°C	Colt & Tchobanoglous (1976)
Carp *Cyprinus carpio*	2.2		Hasan & MacIntosh (1986)
Grass carp *Ctenopharyngodon idelia*	1.61		Zhou et al. (1986)
Fathead minnows *Pimephales promelas*	1.21	pH 9	Thurston et al. (1983)
" "	0.2	pH 6.5	" "
Rainbow trout *Oncorhynchus mykiss*	0.7	DO 8.6 mg L^{-1}	Thurston et al. (1981b)
" "	0.32	DO 2.6 mg L^{-1}	" "
" "	0.53	pH 9	Thurston et al. (1981a)
" "	0.13	pH 6.5	" "

Several acute toxicity studies have incorporated histological observation of gill tissue to examine ammonia induced structural changes in the gills, in an attempt to determine the primary toxic mechanism. Lin and Liu (1990) observed epithelial lifting, hyperaemia and telangiectasis of gill lamellae in hybrid tilapia *Oreochromis mossambicus* x *O. niloticus* exposed to 1.28-2.55 mg L^{-1} UAN for 96 h and concluded that the gill damage observed was characteristic of ammonia intoxication. Morgan and Tovell (1973) suggested that the increased diffusion distance which results from epithelial lifting may slow diffusion and therefore be a protective mechanism. Conversely, Smart (1976) observed relatively minor structural damage to gills of rainbow trout *Oncorhynchus mykiss* exposed to acutely lethal concentrations of ammonia and concluded that gill damage was not a primary cause of acute toxicity of ammonia. In addition, some ammonia toxicity studies have included observations of fish behaviour which have indicated some degree of neurological dysfunction (Daoust and Ferguson, 1984; Lumsden et al., 1993). Other authors have suggested that high concentrations of external ammonia disrupt the Na^+ balance and produce acidosis (Cameron and Heisler, 1983). These may be alternate, or contributing, mechanisms of ammonia toxicity.

The present study was undertaken to determine the short-term toxicity of ammonia to silver perch. Gill histopathology was observed to determine if short-term exposure to high concentrations of ammonia resulted in structural changes to gill tissue.

Materials and Methods

Fish

Prior to stocking the experiment, wet weight of a randomly selected sub-sample of fish was determined to be 2.36±0.09 g; range 1.92 to 3.64 g (x±s.e., n=28). Ten fish were stocked via random interspersion into experimental aquaria and allowed to acclimate to experimental conditions for 5 days. During acclimation, fish in each aquarium were fed as described in Chapter 2 - General Materials and Methods. Two mortalities occurred during acclimation. Dead fish were replaced with fin-clipped fish of a similar size. All aquaria were siphoned prior to the introduction of toxicant. Fish were not fed during the experiment.

Experimental Procedures

Eighteen 100 L fibreglass aquaria were established and maintained on continuous flow throughout acclimation and the experiment as described in Chapter 2 - General Materials and Methods. Concentrations tested were as listed in Tables 3 and 4 (TAN and UAN respectively). The experiment was terminated after 96 h exposure to toxicant.

Water Quality

Ammonia concentration was determined daily according to the method of Dal Pont et al. (1974). After the addition of Analytical Reagent grade NH_4Cl, the pH of the

TABLE 3

Total ammonia - nitrogen (TAN; mean and standard error; n=12) during 96 h experiment

Nominal TAN conc ($mg\ L^{-1}$)	Measured TAN conc ($mg\ L^{-1}$)	se	pH	se
0	0.15	0.02	8.02	0.02
5	7.3	0.09	7.96	0.02
15	15.8	0.19	7.91	0.03
20	23.1	0.24	7.85	0.01
30	33.9	0.74	7.85	0.03
40	40.7	0.45	7.80	0.02

TABLE 4

Un-ionised ammonia (UAN; mean and standard error; n=12) during 96 h experiment

Nominal UAN conc ($mg\ L^{-1}$)	Measured UAN conc ($mg\ L^{-1}$)	se
0	0.009	±0.001
0.4	0.39	±0.02
0.7	0.75	±0.03
1.0	1.0	±0.03
1.4	1.40	±0.05
1.7	1.48	±0.12

header tanks was adjusted using 1M HCl to achieve pH 8 for each aquarium. Temperature, pH and DO for each aquarium were recorded daily. Nominal and actual total ammonia-nitrogen (TAN) concentrations are presented in Table 3. Un-ionised ammonia-nitrogen (UAN) concentrations (nominal and actual) are presented in Table 4. Other water quality variables were within optimum ranges for silver perch. Water temperature varied between 25.7-26.9°C, pH was maintained between 7.8-8.1, DO varied between 5.3-6.1 mg L^{-1}, and alkalinity and hardness, measured at the completion of the experiment, were 85 mg L^{-1} and 75 (as $CaCO_3$ mg L^{-1}) respectively. Salinity was maintained at 0‰

Histology

Histological material was prepared as described in Chapter 2 - General Materials and Methods.

Results

No loss of the ability to orientate was recorded for fish exposed to up to 1.0 mg L^{-1} UAN, while 80% of fish exposed to 1.4 mg L^{-1} UAN and 100% of fish exposed to 1.48 mg L^{-1} UAN lost the ability to orientate and were removed from experimental aquaria (Table 5). Loss of the ability to orientate in fish exposed to 1.4 and 1.48mg L^{-1} UAN commenced after 45 h exposure to toxicant. All fish exposed to 1.48 mg L^{-1} UAN had lost the ability to orientate and the treatment was terminated after 86 h. Prior to loss of the ability to orientate, fish exhibited erratic swimming behaviour. The estimated LC_{50} is approximately 1.2 mg L^{-1} UAN (Fig 1).

TABLE 5

Time of loss of ability to orientate of fish during 96 h ammonia experiment

Time (h)	UAN conc (mg L^{-1})				
0	0.4	0.8	1.0	1.4	1.5
45				8	12
46				1	
48				2	4
49					1
50				2	
69				4	6
70					3
72				1	2
76					1
80				1	
82				1	
86					1
Total				20	30
%				80	100

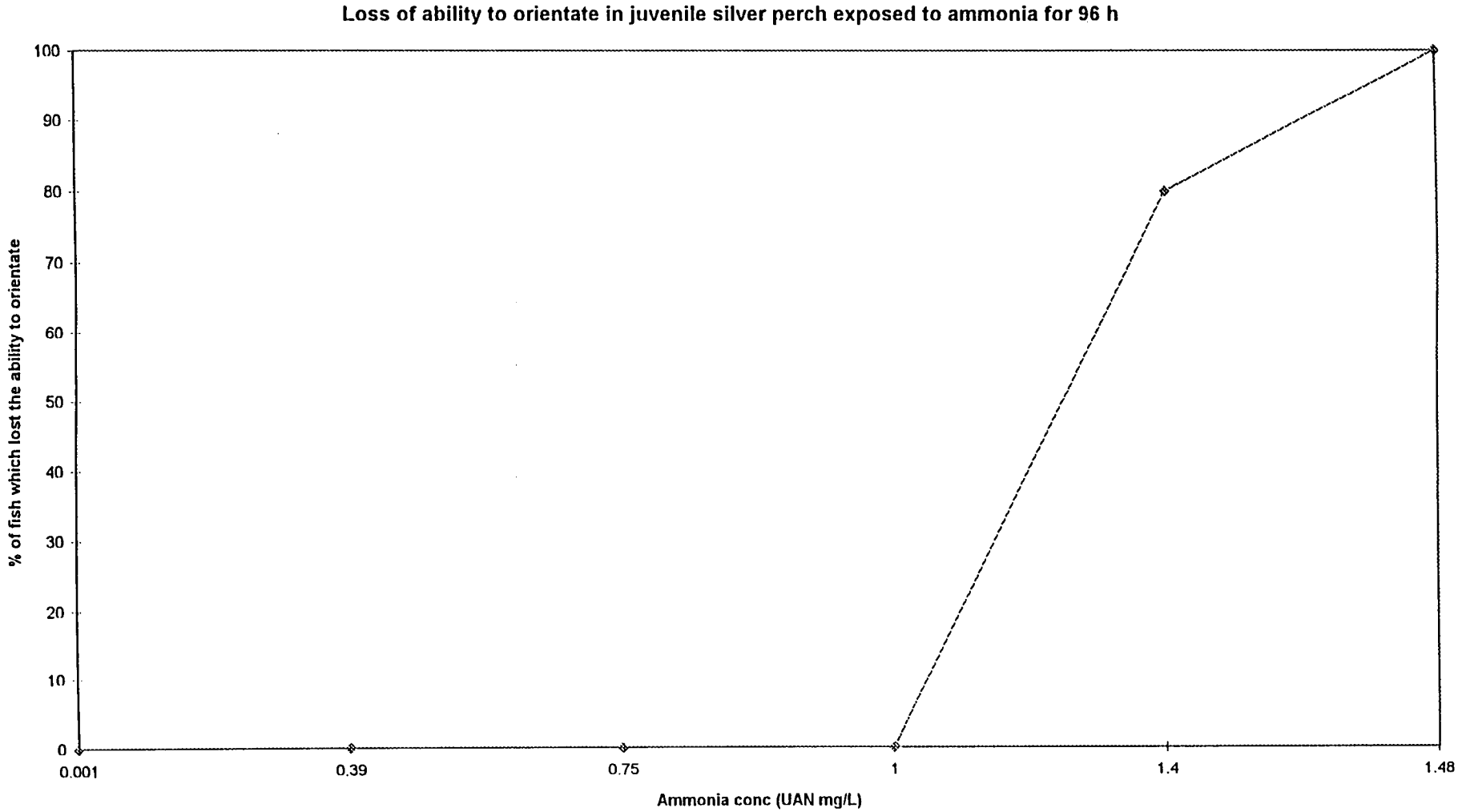

Figure 1 Loss of ability to orientate in juvenile silver perch exposed to ammonia for 96 h

No difference in gill histology was observed between control fish and those exposed to 0.39 mg L^{-1}. The surface of lamellar epithelium was regular (Plate 1 - control). Fish exposed to ammonia concentrations equal to or higher than 0.75 mg L^{-1} UAN showed a significantly ($P<0.05$) higher percentage of filaments affected by epithelial lifting (Table 6; Plate 2 - 1.4 mg L^{-1} UAN). This lifting appears to have resulted from the separation of the two layers of lamellar epithelium, rather than complete separation of the epithelial layer from the pillar cells. No other histopathological changes to gill tissue were observed.

Discussion

Lethal concentrations of ammonia have been determined for many freshwater fish species (see Table 2 and reviews of Russo and Thurston, 1991 and Russo, 1992). The estimated 96h LC_{50} for silver perch (1.2 mg L^{-1} UAN) was within the range of published LC_{50} for other freshwater species (0.13-3.8 mg L^{-1} UAN; Table 2) and indicates that silver perch are moderately resistant to UAN, compared to other species. Table 2 indicates that silver perch are more resistant to ammonia toxicity than are rainbow trout *O. mykiss*, but are similarly susceptible compared with other warmwater species, such as carp *Cyprinus carpio*. A wide variety of physical and chemical factors impact on the toxicity of ammonia to freshwater fish, and differences in these factors between laboratories and over time, together with species-specific differences, contribute to the variability found in published results.

There are some discrepancies in the literature regarding the influence of pH on ammonia toxicity (see reviews of: Meade, 1985; Russo and Thurston, 1991;

Plate 1 Control fish from 96 h ammonia experiment showing regular surface of lamellar epithelium (x 400 magnification).

Plate 2 Epithelial lifting in fish exposed to 1.4 mg L^{-1} UAN for 96 h (x 400 magnification).

100um

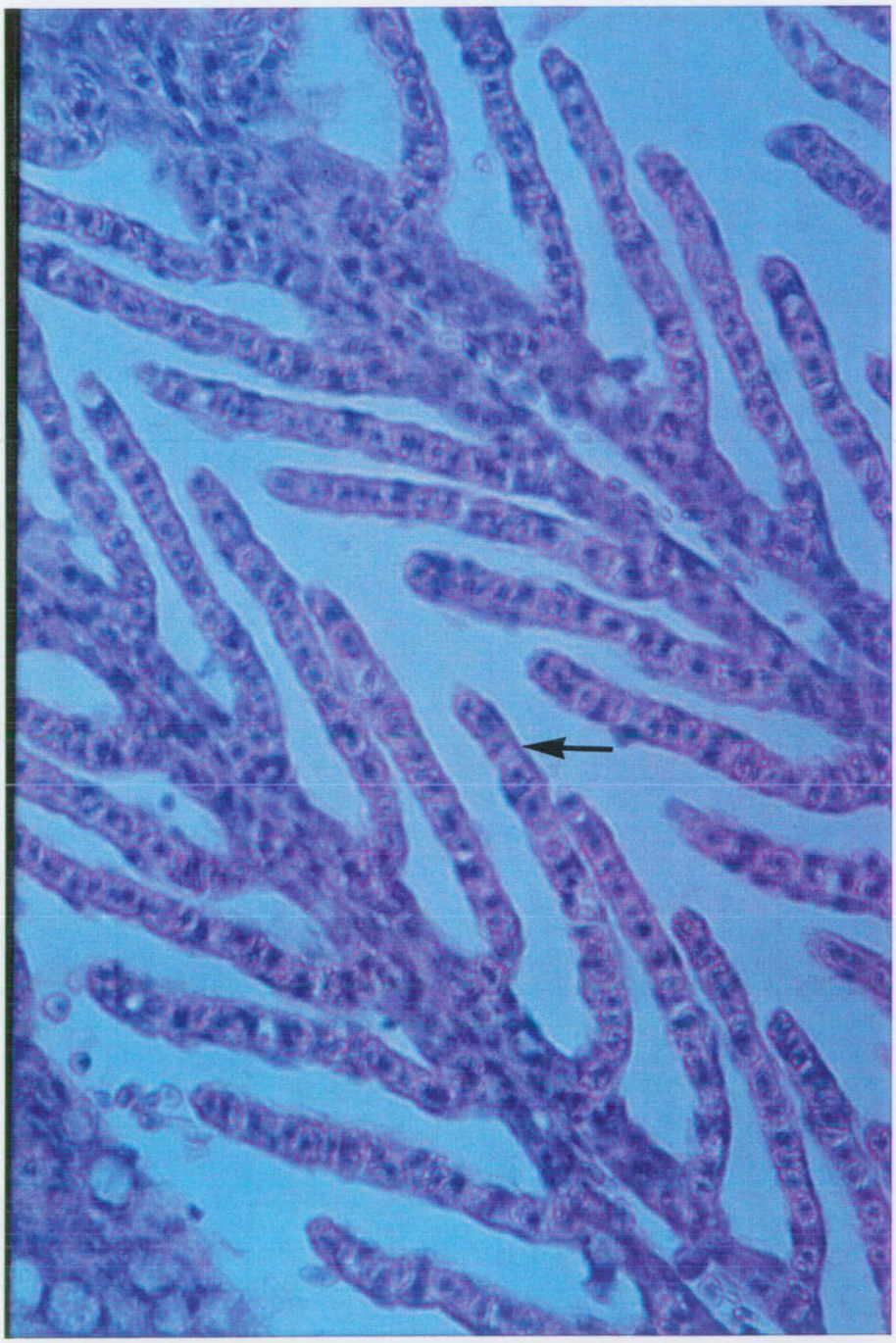

Plate 1

100um

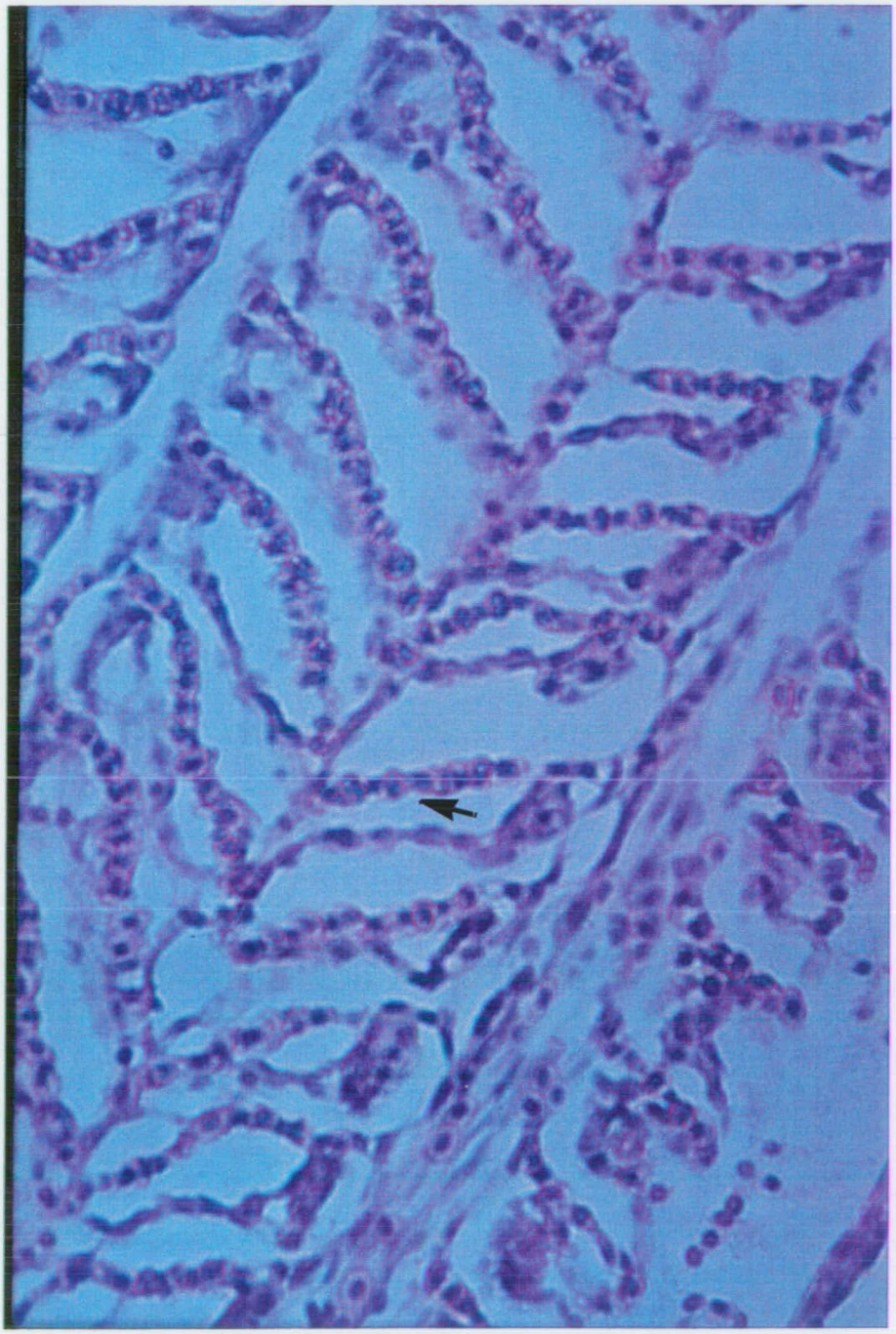

Plate 2

TABLE 6

Percentage of gill filaments showing epithelial lifting for silver perch exposed to ammonia for 96 h

UAN (mg L^{-1})	Epithelial lifting (%)[1,2,3]
0.009	19.2±3.9[a]
0.39	29.2±5.7[a]
0.75	46.0±5.4[b]
1.0	49.0±4.7[b]
1.4	51.9±5.2[b]
1.48	54.8±4.9[b]

1 Values are means ± se

2 Within columns, different letters in the superscript indicate a significant difference

3 Data were subjected to arcsine transformation prior to analysis

Tomasso, 1994). In the present study, pH was adjusted to 8, with variation from 7.8 to 8.1. The highest degree of pH variability (Table 3) was recorded for those fish exposed to the highest concentration of UAN (Table 4), due to the depressing effect of the addition of NH_4Cl on pH. The effect of subtle pH variations on ammonia toxicity has not been established for silver perch, and evidence from published studies varies such that an expected trend cannot be anticipated. Tomasso et al. (1980) found the 24 h LC_{50} of UAN for channel catfish *Ictalurus punctatus* at pH 8 to be significantly higher than at pH 7 or 9, while Thurston et al. (1981a) found the increase in ammonia toxicity for rainbow trout *O. mykiss* at increasing pH could not solely be attributed to an increased proportion of UAN present. Sousa et al. (1974) found the resistance of chinook salmon *Oncorhynchus tshawytscha* smolts to ammonia toxicity was increased by mildly acidic culture conditions. The optimal pH for silver perch has not been established, although the recommended pH range (6.5 to 9.0; Rowland [1995b]) for pond culture indicate that silver perch are tolerant of a wide range of pH conditions. An understanding of the influence of pH on the toxicity of ammonia to silver perch would be useful to aquaculturists.

Temperature also affects the position of the ammonia dissociation equilibrium, with increasing temperature favouring an increasing proportion of total ammonia present as UAN (Emerson et al., 1975; Ferguson, 1988). A 10°C increase in temperature doubles the concentration of UAN present in an ammonia solution (EIFAC, 1970). However, some studies report ammonia to be less toxic at temperatures approaching optimal for a given species (Colt and Tchobanoglous, 1976; Thurston and Russo, 1983). Thurston et al. (1983) found the acute toxicity of UAN to fathead minnows *Pimephales promelas* decreased as temperature increased between 12 and 22°C.

Further, Rosenboom and Richey (1977) reported similar trends for UAN toxicity to bluegill *Lepomis macrochirus*, channel catfish *I. punctatus* and largemouth bass *Micropterus salmoides*. Ministry of Technology (1968) found a threefold increase in the 24 h LC_{50} for rainbow trout *O. mykiss* from 5 to 18°C. The present study was conducted at 25.7-26.9°C, which is considered to be in the optimal range for silver perch (Rowland, 1995b). It therefore appears reasonable to deduce that 1.2 mg L^{-1} UAN is close to the maximum 96 h tolerance for silver perch at any temperature.

Elevated ammonia levels have been shown to impair the oxygen carrying capacity of the blood (Brockway, 1950), and several studies have established an inverse relationship between ammonia toxicity and dissolved oxygen (DO) for salmonids. Alabaster et al. (1979) found the toxicity of UAN to Atlantic salmon *Salmo salar* smolts was increased at low DO, and Downing and Merkens (1955) found the susceptibility of rainbow trout *O. mykiss* to UAN decreased as DO was raised from 1.5 to 8.5 mg L^{-1}. In work with the same species, Lloyd (1961) showed ammonia toxicity to be elevated by decreasing DO and suggested that this may result from the increased respiratory rate and hence increased volume of water passing over the gills. In studies of rainbow trout *O. mykiss*, perch *Perca fluviatilis*, roach *Rutilus rutilus* and gudgeon *Gobio gobio*, Merkens and Downing (1957) found decreasing DO to increase the toxicity of UAN to all species studied, with the exception of gudgeon, which showed no significant difference in susceptibility. Thurston et al. (1981b) found UAN toxicity to rainbow trout *O. mykiss* increased as DO decreased from 8.6 to 2.6 mg L^{-1} and was more strongly correlated for short-term exposures. These authors estimated the UAN tolerance at 5 mg L^{-1} DO to be 30% less than at 8.5 mg

L^{-1} DO. However, Thurston et al. (1983) found no significant relationship between DO and ammonia toxicity to fathead minnows *P. promelas* between 3 and 9 mg L^{-1} DO. Dissolved oxygen concentrations in the present study were maintained at 6.4 to 7.2 mg L^{-1}, representing 80% and 90% saturation, respectively, and DO was therefore unlikely to impact on ammonia toxicity in the present study.

Gill histology has often been observed following ammonia toxicity studies to examine mode of toxic action. Some published studies have established damage to gill tissue following acute exposure to ammonia. Tarazona et al. (1987) observed gill congestion and haemorrhage in goldfish *Carassius auratus* following acute exposure to 0.91 mg L^{-1} UAN. Daud et al. (1988) found gross gill filament haemorrhage in red tilapia *O. mossambicus* x *O. niloticus* following seven days' exposure to 3.4 mg L^{-1} UAN, however, histological observations were not undertaken. Histopathological changes, notably haemorrhage and telangiectasis were present in gills of tilapia *Tilapia aurea* following exposure to 2.35 mg L^{-1} UAN for 48 hours (Redner and Stickney, 1979). Furthermore, Smart (1976) observed severe histopathological changes to gill tissue of rainbow trout following long-term exposure to ammonia but not short-term. The apparent disparity between the extent and severity of histopathological changes observed between studies indicates that the response mechanism may be species-specific. In addition, differences in other water quality variables between laboratories may have interacted with ammonia and contributed to observed changes.

In the present study, the percentage of filaments affected by epithelial lifting was significantly ($P<0.05$) increased by short-term exposure to ammonia (Table 6).

Epithelial lifting increases the diffusion distance across the respiratory epithelium (Mallatt, 1985), resulting in less efficient gas exchange, which may impact on respiratory processes and may compromise the ability of fish to obtain oxygen. A significant increase in the percentage of filaments affected by epithelial lifting was observed in fish exposed to UAN concentrations greater than 0.8 mg L^{-1} UAN (Plate 2), while the ability to orient was not significantly impacted for those fish exposed to 1.0 mg L^{-1} UAN (Table 5). This indicates that epithelial lifting may be a preliminary indicator of sub-lethal intoxication. In addition, the epithelial lifting observed in the present study may be a protective mechanism against further intoxication, as suggested by Morgan and Tovell (1973). Similarly, Lin and Liu (1990) found an increase in the percentage of filaments affected by epithelial lifting in hybrid tilapia (*O. mossambicus* x *O. niloticus*) exposed to 1.69 mg L^{-1} UAN for 12 h. However, in the present study no other statistically significant histopathological changes to gill tissue were observed. The absence of other histopathological damage suggests that gill damage was relatively minor and may not be the primary mechanism of acute ammonia intoxication in silver perch. Similarly, Smart (1976) found little evidence of gill damage in rainbow trout *O. mykiss* exposed to acutely toxic concentrations of ammonia and suggested that gill damage was not the primary cause of death. Ferguson (1989) stated that morphological change probably lags far behind functional impairment. It is unlikely that the epithelial lifting observed in the present study was sufficiently severe to cause the loss of equilibrium recorded in fish removed from experimental conditions and sampled for histological preparations. A toxic mechanism, other than gill histopathology, is suggested.

A commonly observed effect of ammonia toxicity, particularly following acutely toxic UAN insult, is the occurrence of brain lesions and associated neurological dysfunction. Lumsden et al. (1993) observed paralysis in farmed Arctic char *Salvelinus alpinus* and suggested that coma may have resulted from interference with neurotransmittters in the brain. Smart (1978) exposed rainbow trout *O. mykiss* to acute UAN concentrations and suggested that the toxic reaction resulted from impairment of cerebral energy metabolism. Likewise, Daoust and Ferguson (1984) observed signs suggestive of neurological disorder in rainbow trout *O. mykiss* exposed to 0.4 mg L^{-1} UAN for four days. Histological examination of gill tissue from that study did not indicate gill lesions. The silver perch in the present study exhibited erratic swimming behaviour prior to the loss of ability to orientate. Such behaviour may be associated with ammonia-induced neurological dysfunction.

The results of this study indicates that the estimated 96 h LC_{50} is approximately 1.2 mg L^{-1} UAN for juvenile silver perch. The threshold concentration at which epithelial lifting was significantly increased was lower than that at which the ability to orient was affected. Therefore, epithelial lifting may be considered a preliminary indicator of acute ammonia toxicity in juvenile silver perch. However, as epithelial lifting was the only histopathological effect observed to increase with exposure to un-ionised ammonia, it is concluded that general damage to gill tissue is not the primary mechanism of acute ammonia toxicity in silver perch. Behavioural observations of fish prior to their removal from the experiment (including erratic swimming behaviour and loss of equilibrium) indicate that cerebral dysfunction may be involved in the toxic action of ammonia. Further studies are recommended.

CHAPTER 4

The Acute Toxicity of Nitrite to Silver Perch

Introduction

The sensitivity of freshwater fish to acute exposure to nitrite varies widely between species (see Table 7 and reviews of Lewis and Morris [1986], Russo and Thurston [1991] and Russo [1992]). Water chemistry has been found to influence resistance to nitrite intoxication, particularly chloride concentration (Perrone and Meade, 1977; Mazik et al.,1991), hardness (Wedermeyer and Yasutake, 1978) and DO (Bowser et al., 1983; Watenpaugh and Beitinger, 1986). Other factors affecting susceptibility to acute nitrite concentrations include genetic variability (Tomasso and Carmichael, 1991) and the possession of a nitrite exclusion mechanism (Palachek and Tomasso, 1984a).

Knowledge of the concentration of nitrite which is acutely toxic to a species of interest to commercial aquaculture is of great importance to maximise production in that industry. Interest in the farming of silver perch is increasing (Rowland, 1995a), however, the concentration of nitrite which is acutely toxic to silver perch is yet to be established.

Several acute toxicity studies have incorporated histopathological evidence to elucidate the toxic mechanism and reveal possible toxicant-induced structural changes. Through observation of gill tissue preparations, Krous et al. (1982)

TABLE 7

The 96 h LC_{50} of nitrite to freshwater fish

Species	96 h LC_{50} (mg L^{-1} NO_2-N)	Reference
Striped bass *Morone saxatilis*	160	Mazik et al. (1991)
Largemouth bass *Micropterus salmoides*	140.2	Palachek & Tomasso (1984a)
Tilapia *Tilapia aurea*	28	Hilmy et al. (1987)
" "	16.2	Palachek & Tomasso (1984a)
Sunshine bass *Morone chrysops* x *M. saxatilis*	12.8	Weirich et al. (1993)
Channel catfish *Ictalurus punctatus*	12.8	Colt & Tchobanoglous (1976)
" "	7.1	Palachek & Tomasso (1984a)
Fathead minnow *Pimephales promelas*	2.99	Russo & Thurston (1977)
Chinook salmon *Oncorhynchus tshawytscha*	0.88	Westin (1974)
Cutthroat trout *Salmo clarki*	0.5-0.6	Thurston et al. (1978)
Rainbow trout *Oncorhynchus mykiss*	0.19-0.39	Russo et al. (1974)

believed lamellar chloride cells facilitated the transport mechanism for nitrite in rainbow trout *Oncorhynchus mykiss*, while Arillo et al. (1984), in studies of the same species, observed ultrastructural changes to the livers following exposure to 450 ug L^{-1} NO_2-N for 96 hours and suggested such damage was the root of the acute toxicity mechanism. Other authors have utilised haematological techniques to investigate blood nitrite levels (Margiocco et al., 1983) and/or determine haemoglobin and methemoglobin concentrations (Brown and McLeay, 1975; Scarano and Saroglia, 1984; Almendras, 1987) in an attempt to determine the mode of toxic action.

The present study was undertaken to determine the short-term toxicity of nitrite. Gill histopathology was observed to determine if short-term exposure to nitrite resulted in structural changes to gill tissue.

Materials and Methods

Fish

Fish were sourced as described in General Materials and Methods.

Prior to stocking the experiment, wet weight of a randomly selected sub-sample of fish was determined to be 6.7±1.3 g; range 4.2 to 8.8 g (x±s.e., n=20). Ten fish were stocked via random interspersion into experimental aquaria and allowed to acclimate to experimental conditions for 5 days. During acclimation, fish in each aquarium were fed as described in Chapter 2 - General Materials and Methods. Dead fish were

replaced with fin-clipped fish of a similar size. All aquaria were siphoned prior to the introduction of toxicant. Fish were not fed during the experiment.

Experimental Procedures

Eighteen 100 L fibreglass aquaria were established and maintained as described in Chapter 2 - General Materials and Methods. The header tanks were dosed with Analytical Reagent grade sodium nitrite to achieve the nominal concentrations 0, 35, 65, 100, 135 and 165 mg L^{-1} $N0_2$-N (nominal and actual $N0_2$-N concentrations are listed in Table 8).

This experiment was conducted following limit-testing procedures, as described in the General Materials and Methods. The experiment was terminated after 96 h exposure to toxicant.

Water Quality

Nitrite concentration, temperature, pH, DO and salinity was measured and recorded daily as described in the General Materials and Methods. Nominal and actual nitrite concentrations are presented in Table 8. Other water quality variables were within optimum ranges for silver perch. Water temperature varied between 25.1-26.2°C, pH was maintained between 7.8-8.3, DO varied between 6.4-7.2 mg L^{-1}, and alkalinity and hardness, measured at the completion of the experiment, were 90 mg L^{-1} and 75 (as $CaCO_3$ mg L^{-1}) respectively. Salinity was maintained at 0‰

TABLE 8

Nitrite (NO_2-N) concentrations during 96 h nitrite experiment

Nominal nitrite conc (mg L^{-1})	Measured nitrite conc (mg L^{-1})	se
0	0.001	± 0.0
35	35.2	± 1.5
65	65.4	± 3.4
100	99.8	± 6.0
135	135.8	±10.6
165	179.4	± 7.8

Histology

The gills of 5 fish (where possible) from each aquarium were sampled for histological examination, as described in the General Materials and Methods.

Results

Erratic swimming behaviour was observed in fish prior to loss of ability to orientate. Fish which lost the ability to orientate were removed from experimental aquaria. Fish did not lose the ability to orientate until the treatment concentration exceeded 35 mg L^{-1} NO_2-N. The time at which fish were removed from the experiment is presented in Table 9. The estimated 96 h LC_{50} was approximately 160 mg L^{-1} NO_2-N (Fig 2).

In general, gross structure of gill tissue appeared normal, however, those fish exposed to 130 mg L^{-1} $N0_2$-N exhibited definite browning and discolouration of gill tissue at the time of histological sampling. Negligible histopathological changes were recorded for all samples within all treatments. Plate 3 illustrates gill tissue of control fish, while Plate 4 depicts gill tissue from fish exposed to 179 mg L^{-1} $N0_2$-N.

Discussion

Lethal concentrations of nitrite have been determined for many freshwater species, generally through 96h LC_{50} studies. Results from published acute nitrite studies indicate that the tolerance of fish to nitrite varies widely (Table 8). Nitrite toxicity is

TABLE 9

Time of loss of ability to orientate of fish during 96 h nitrite experiment

Time (h)	NO_2-N conc (mg L^{-1})				
	0	35	65	100	136	179
22						1
28				1		1
45				1	5	7
50					2	4
60						1
69			1		2	1
94						2
Total			1	2	9	17
%			3	7	30	57

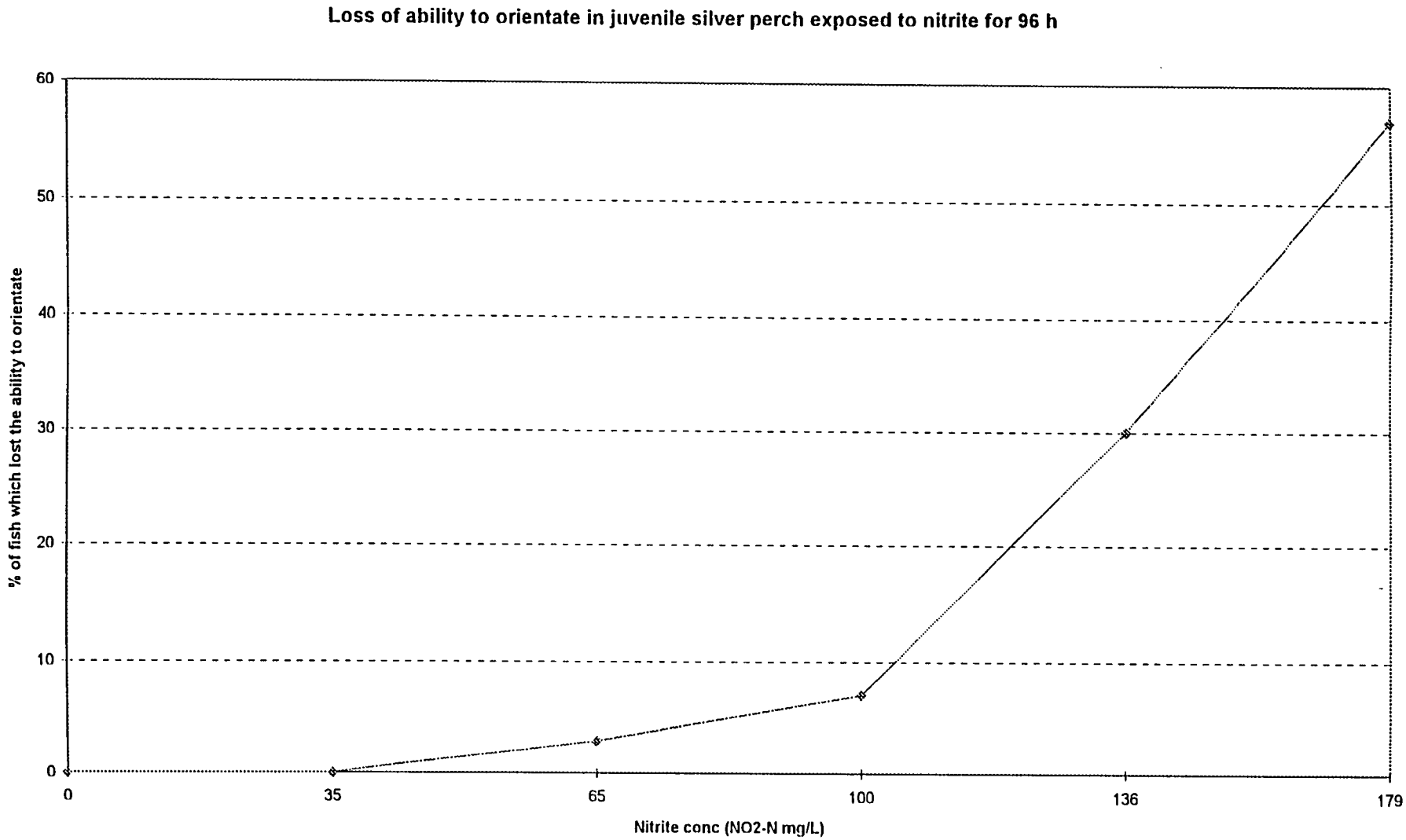

Figure 2 Loss of ability to orientate in juvenile silver perch exposed to nitrite for 96 h

Plate 3 Control fish from 96 h nitrite experiment (x 100 magnification).

Plate 4 Fish exposed to 179 mg L^{-1} NO_2-N for 96 h (x100 magnification).

100um

Plate 3

100um

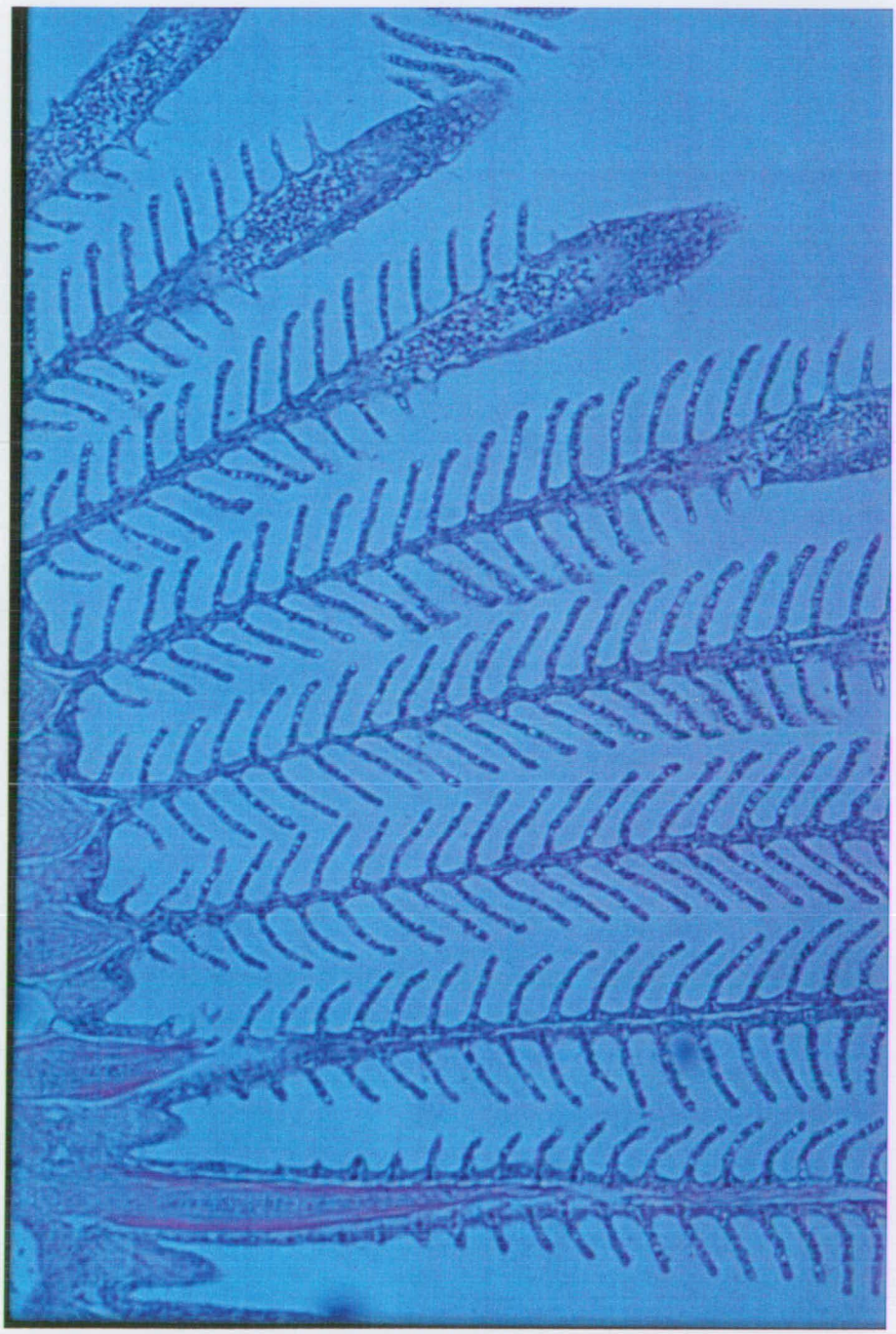

Plate 4

clearly species-specific. Genetic variability within strains and differences between strains have been shown to impact on nitrite toxicity in channel catfish *Ictalurus punctatus* (Tomasso and Carmichael, 1991). In addition, the toxicity of nitrite to a variety of freshwater species has been shown to be affected by other water quality variables, notably salinity (Perrone and Meade, 1977), dissolved oxygen (DO; Bowser et al. 1983) and hardness (Wedermeyer and Yasutake, 1978).The present study reveals that silver perch are relatively resistant to acute exposure to nitrite.

Resistance to nitrite toxicity has been established for some other species. Largemouth bass *Micropterus salmoides* do not concentrate nitrite in the blood at levels higher than environmental concentrations, which indicates the possession of an exclusion mechanism which imparts resistance to nitrite intoxication (Palachek and Tomasso, 1984a). In the same study, channel catfish *I. punctatus* and tilapia *Tilapia aurea* showed increased methemoglobin levels with increasing environmental nitrite, while the methemoglobin levels of largemouth bass *M. salmoides* did not increase until the environmental nitrite concentration was more than double the 96 h LC_{50} of the other two species in that study. The resistance of silver perch to acute exposure to nitrite in the present study was similar to that of largemouth bass *M. salmoides* in Palachek and Tomasso's (1984a) study, indicating that silver perch may also possess a nitrite exclusion mechanism.

Other species have been found to actively concentrate nitrite in the blood. Margiocco et al. (1983) showed rainbow trout *O. mykiss* with a blood nitrite concentration up to 60 times higher than environmental concentrations. Several studies have suggested that some species may be unable to distinguish between Cl^-

and NO_2^- ions (Bath and Eddy, 1980; Meade and Perrone, 1980). Freshwater fish possess a chloride active transport mechanism to maintain ionic balance (Love, 1980), and therefore an inability to distinguish between these ions at the cell membrane (Eddy et al., 1983) would account for the observation of blood nitrite concentrations higher than environmental nitrite concentrations. One possible mechanism for the apparent resistance of silver perch to short-term exposure to relatively high concentrations of nitrite could be the ability to distinguish between Cl^- and NO_2^- ions at the cell membrane.

Polished bore water was used in the present study, with a hardness and alkalinity content of 75 mg L^{-1} and 90 mg L^{-1}, respectively. Wedermeyer and Yasutake (1978) found a large reduction in the acute toxicity of nitrite to steelhead trout *Oncorhynchus mykiss* when total water hardness was increased from 25 to 3 000 mg L^{-1}. It is possible that a similar reduction in the acute toxicity of nitrite to other freshwater species would also be brought about by such an increase in water hardness, however this is yet to be established for silver perch.

A well documented effect of nitrite is its ability to convert haemoglobin to methemoglobin, a form unable to carry oxygen (Brown and M^{c}Leay, 1975, Wedermeyer and Yasutake, 1978). The resultant condition is known as methemoglobinaemia. It therefore appears possible that the silver perch fingerlings in this study suffered from methemoglobinaemia to some extent. Under high nitrite concentrations, the reduction of haemoglobin to methemoglobin has often been noted to cause the blood and gills to brown and darken (Smith and Williams, 1974; Smith and Russo, 1975; Huey et al., 1980; Scarano et al., 1984), and is therefore commonly

referred to as 'brown blood disease' (Konikoff, 1975). In the present study, those fish exposed to 130 mg L^{-1} NO_2-N and above showed definite browning and discolouration of gill tissue which was observed macroscopically during histological sampling.

Methemoglobinaemia is said to compromise a fish's ability to tolerate low dissolved oxygen conditions (Bowser et al., 1983; Watenpaugh and Beitinger, 1986). As ectotherms, there is a direct relationship between fish activity and environmental temperature, though optimal temperatures are species-specific. Metabolic requirements for oxygen are affected by temperature (Cairns et al., 1975), and as nitrite reduces the oxygen carrying capacity of blood, it appears likely that temperature and dissolved oxygen will influence nitrite toxicity. However, studying channel catfish *I. punctatus,* Colt and Tchobanoglous (1976) found no relationship between nitrite toxicity and temperature, but found the toxicity of nitrous acid increased with increasing temperature. Working with the same species, Huey et al. (1984) found methemoglobin concentrations of nitrite-exposed fish at 30°C to be almost double that of those at 10°C. These authors went on to suggest that fish may survive severe methemoglobinaemia by reducing their oxygen requirement through behaviour modification and by using oxygen dissolved in plasma. Nitrite exposure may impart a reduced capacity for sustained activity (Huey et al., 1984), due to its effects on the oxygen carrying capacity of the blood. Similarly, Watenpaugh and Beitinger (1986) suggested that channel catfish *I. punctatus* minimised activity and therefore decreased oxygen consumption rates following exposure to nitrite-induced hypoxia. The present study was conducted at temperatures between 25.1°C and 26.2°C , which are considered near optimal for growth of silver perch (Rowland,

1995b). The dissolved oxygen concentration was maintained at between 6.4 and 7.2 mg L^{-1} in the present study, sufficient to maintain normal activity in nitrite-free water. It is possible that silver perch in the present study utilised a behaviour modification to reduce activity levels and preserve limited blood oxygen supplies. As the fish in the present study were unfed during the experiment, this may have reduced reasons for fish activity (ie procurement and competition for food).

There was no difference in gill histopathological changes with exposure to increasing concentrations of nitrite (see Plates 3 and 4). Similarly, Perrone and Meade (1977) found no evidence of histopathological damage to gill tissue of coho salmon *Oncorhynchus kisutch* exposed to up to 30 mg L^{-1} NO_2-N for 96 h. The findings of the present study indicate that physical damage to the fine structure of gills of silver perch does not occur during 96 h exposure to high concentrations of nitrite, and that therefore gill damage is not a primary mechanism in the acute toxicity of nitrite to silver perch.

In conclusion, this study establishes that the estimated 96 h LC_{50} is approximately 160 mg L^{-1} NO_2-N. Gill histopathological damage is not a primary mechanism for acute nitrite toxicity in silver perch. Browning and discolouration of gill tissue was observed macroscopically, indicating methemoglobinaemia, which may be the primary mechanism of acute toxicity of nitrite to juvenile silver perch. Further studies incorporating haematological observations and an assessment of methemoglobin production with nitrite toxicity are recommended.

CHAPTER 5

The Short-Term Growth-Limiting Concentration of Ammonia for Silver Perch

Introduction

Elevated levels of ammonia can cause growth reduction in freshwater fish, including channel catfish *Ictalurus punctatus* (Colt and Tchobanoglous, 1978) and rainbow trout *Oncorhynchus mykiss* (Smith and Piper, 1975; Soderberg et al., 1983). The concentration at which growth is inhibited is species-specific and exposure to concentrations between 0.002-0.27 mg L^{-1} UAN have caused growth reduction in a number of species (Russo and Thurston, 1991; Russo, 1992).

The mode of action responsible for ammonia toxicity has been widely studied. Gill histology observations have often been associated with ammonia toxicity studies in an attempt to elucidate mode of action (Mallatt, 1985). Robinette (1976) observed hyperplastic tissue changes to gills of channel catfish *I. punctatus* fingerlings following exposure to sublethal ammonia concentrations, while rainbow trout *O. mykiss* chronically exposed to 0.7 mg L^{-1} UAN for eight months in culture facilities developed severe hyperplasia and aneurysm in gills (Larmoyeux and Piper, 1973). Conversely, Daoust and Ferguson (1984) found no gill lesions attributable to ammonia in rainbow trout *O. mykiss* following a 90 day exposure to 0.4 mg L^{-1} UAN. More recently, Kirk and Lewis (1993) found ammonia intoxication resulted in the occurrence of distinctive circular depressions in gill epithelium and deformation of the gill filament and lamellae of rainbow trout *O. mykiss* and recommended the use

of scanning electron microscopy (SEM) as a diagnostic tool. Differences in toxic action of ammonia sourced from "off-the-shelf" reagents and that arising from exposure to ammonia of metabolic origin is a possible explanation for the disparity in published results (See Table 1 in Chapter 1 - Introduction in this thesis).

Other organs have been investigated to determine the mode of action of ammonia intoxication, including brain and nervous system (Fromm and Gillette, 1968; Smart, 1978; Thurston et al., 1986; Lumsden et al., 1993), kidney (Daoust and Ferguson, 1984; Lightner et al., 1988) and liver (Soderberg et al., 1984; Soderberg, 1985). In addition, changes in blood chemistry resulting from exposure to ammonia have also been investigated (Cameron and Heisler, 1983; Wlasow and Dabrowska, 1990; Jeney et al., 1992a, 1992b).

Knowledge of the concentration of ammonia which limits growth is useful for aquaculture management and may assist in the maximisation of production (Colt and Armstrong, 1981). In addition, silver perch often exhibit "off-flavours" as a result of the presence of blue-green algae and/or actinomycetes in the earthen ponds in which they are cultivated (Rowland, 1995c). Prior to marketing, harvested live silver perch require purging in clean water for five to fifteen days to eliminate off-flavours (Rowland, 1995c). This process is generally undertaken in fibreglass tanks which may be static or recirculating. Holding large fish in tanks with limited water exchange may result in the accumulation of ammonia. Knowledge of the concentration of ammonia which limits growth and/or impairs respiratory function is a useful management tool during purging of silver perch.

The aim of the present study was to determine the growth-limiting concentration of ammonia for juvenile silver perch. The effects of Analytical Reagent ammonia and ammonia of metabolic origin on silver perch fingerlings under laboratory conditions was also compared. Gill histology was examined to determine if ammonia affected silver perch gills and, if so, whether gill structure is a useful indicator of ammonia intoxication. Routine histology was used to identify changes in gill morphology.

Methods

Fish

Fish were sourced as described in General Materials and Methods. Prior to stocking the experiment, fish were graded to exclude fish outside the weight range 1.8-2.2 g. The fish were lightly anaesthetised using 20 mg L^{-1} benzocaine and individually weighed. Ten fish per replicate were then stocked into aquaria via random interspersion (1.99±0.05g; x±se; n=210). Fish were allowed to acclimate to experimental conditions for 7 days, during which fish in each aquarium were fed as described in Chapter 2 - General Materials and Methods. Survival, growth and gill histopathology were the variables used to evaluate the effect of the toxicant.

Experimental Procedures

Concentrated Analytical Reagent grade ammonium chloride solutions were prepared using laboratory test water as diluent and placed into 15 L reservoirs. Eighteen 70 L

aquaria (one control treatment and five test concentrations, each with three replicates) were established as described in Chapter 2 - General Materials and Methods.

A further treatment was established in which fish were exposed to elevated ammonia concentrations of metabolic origin. To achieve high concentrations of metabolic ammonia, prior to the commencement of the experiment large silver perch were held in a 10 000 L tank under crowded conditions. Waste water from this tank was siphoned off and transferred to the experimental laboratory, where a recirculating system was established involving only the 3 replicate aquaria of this treatment.

Concentrations tested were 0.01, 0.03, 0.04, 0.07, 0.14, and 0.36 mg L^{-1} UAN (0.13, 0.29, 0.5, 0.8, 1.7 and 4.3 mg L^{-1} TAN). The concentration achieved in the ammonia of metabolic origin treatment was 0.02 mg L^{-1} UAN (0.21 mg L^{-1} TAN).

The feeding and management of fish and the treatment of uneaten feed is described in the General Materials and Methods. No mortality occurred in any of the control aquaria. The experiment was terminated after 39 days' exposure to toxicant.

Water Quality

Ammonia concentrations were determined daily according to the indophenol method described by Dal Pont et al. (1974). Nitrite concentrations were determined weekly for all reagent ammonia aquaria and daily for all metabolic ammonia aquaria using the method of Major et al. (1972). The pH of source water for reagent ammonia aquaria was adjusted to 8 by the addition of caustic soda to the storage tank.

Dissolved oxygen, temperature, and salinity were measured daily as described in the General Materials and Methods. The pH was measured daily using Metrohm Model 605 pH meter and Metrohm reference and glass electrodes (Metrohm Pty Ltd, Switzerland) and was calibrated daily using NBS phosphate and borate buffers (Chemical Rubber Company, 1971). Alkalinity and hardness were determined at the completion of the experiment using commercial test kits (Aquasonics Pty Ltd, Ingleburn, NSW, Australia). Colourimetric measurements were determined as described in the General Materials and Methods. Values for these variables for reagent ammonia aquaria are presented in Table 10, and for metabolic ammonia aquaria are presented in Table 11. Temperature and dissolved oxygen were maintained at 24.7-27.2°C and 5.4-6.9 mg L^{-1} respectively, and the pH varied from 7.85 to 8.24. Photoperiod was maintained at 12 h light:12 h dark.

Histology

Five fish per aquarium (105 fish in total) were killed using benzocaine (*p* aminobenzoate) overdose at 50mg L^{-1} and sampled for histological preparations as described in Chapter 2 - General Materials and Methods.

Statistical Analysis

Statistical analysis was undertaken as described in the General Materials and Methods. Average weight gain data were subjected to linear regression to determine the concentration at which growth was not affected. The concentration at which growth was reduced by 5% was calculated from this curve.

TABLE 10

Water quality data for Analytical Reagent ammonia aquaria during the ammonia growth-limiting experiment.

	Temp °C	DO mg L^{-1}	pH mg L^{-1}	Nitrite ‰	Salinity (‰)	Alkalinity (as Ca CO_3)	Hardness
x	26.0	6.3	8.10	0.4	0.04	90	75
se	0.015	0.032	0.004	0.02	0.00	-	-
max	27.2	6.9	8.24	1.72	0.05	-	-
min	24.7	5.4	7.85	0.01	0.01	-	-

TABLE 11

Water quality data for metabolic ammonia aquaria during the ammonia growth-limiting experiment.

	Temp °C	DO mg L^{-1}	pH	Nitrite mg L^{-1}	Salinity ‰	Alkalinity (mg L^{-1})	Hardness (as Ca CO_3)
x	26.0	5.9	8.04	1.91	0.04	85	65
se	0.015	0.03	0.00	0.13	0.00	-	-
max	27.2	6.3	8.14	4.24	0.05	-	-
min	24.7	5.4	7.85	0.12	0.01	-	-

Results

Survival

No abnormal behaviour was observed in either control fish or those exposed to ammonia and appetite appeared to be good throughout the experiment. Overall survival was high, with less than 1% mortality. Two mortalities were recorded from one aquaria (0.36 mg L^{-1} UAN) two days prior to the termination of the experiment. Survival in all other aquaria exposed to ammonia and in controls was 100% Fin-clipped replacement fish survived for the remaining course of the experiment.

Growth

Ammonia concentration had a significant effect ($P<0.05$) on wet weight gain and specific growth rate (SGR; Table 12). Ammonia concentration had no significant effect ($P>0.05$) on food conversion ratio (FCR). A significant difference ($P<0.05$) in wet weight gain was observed between control fish and those exposed to 0.36 mg L^{-1} UAN. The EC_5 is 0.06 mg L^{-1} UAN (Fig 3).

Histology

No gross pathology of gill tissue was observed. The filaments of all fish were straight and the lamellae were arranged evenly and parallel. Mucous cells were located distally in all preparations. No chloride cells were observed. A comparison of prepared histological material under light microscope revealed changes to gill

TABLE 12

Wet weight gain, specific growth rate (SGR), food conversion ratio (FCR) and food consumption of silver perch exposed to sub-lethal concentrations of ammonia (mg L^{-1} UAN) for 39 days.

UAN (mg L^{-1})	Wet weight gain (g $fish^{-1}$)[1,2]	SGR[1,2]	FCR[1,2]	Food consumption[1,2]
0.01 ±0.001	4.3±0.3^{a}	2.6±1.1^{a}	2.1±0.2^{a}	9.0±0.8^{a}
0.02 ±0.003 (Metabolic)	3.8±0.3^{ab}	2.4±0.1^{ab}	2.1±0.2^{a}	7.8±0.4^{ab}
0.03 ±0.001	3.8±0.5^{ab}	2.4±0.2^{ab}	1.9±0.1^{a}	7.2±0.8^{ab}
0.04 ±0.001	3.9±0.4^{ab}	2.4±0.1^{ab}	2.0±0.1^{a}	7.9±0.6^{ab}
0.07 ±0.002	3.4±0.1^{ab}	2.2±0.0^{ab}	1.8±0.1^{a}	5.9±0.5^{b}
0.14 ±0.005	3.2 ±0.1^{ab}	2.1±0.0^{ab}	2.2±0.1^{a}	6.8±0.1^{ab}
0.36 ±0.021	2.9 ±0.1^{b}	1.9±0.1^{b}	2.2±0.1^{a}	6.4±0.4^{ab}

1 Values are means ± se

2 Within columns, different letters in the superscript indicate a significant difference ($P<0.05$)

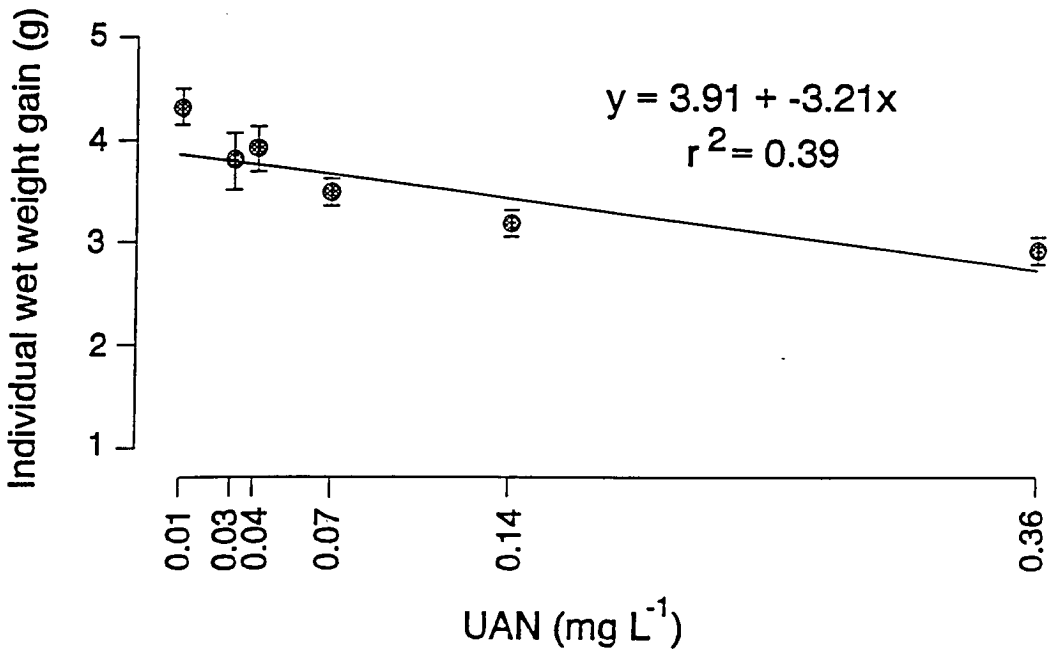

Figure 3 Regression of analysis of individual wet weight gain for juvenile silver perch exposed to ammonia

filaments following exposure to ammonia (Table 13). The most significant change was an increase in the percentage of filaments exhibiting epithelial lifting. Gills of fish exposed to 0.36 mg L^{-1} UAN had a significantly ($P<0.05$) higher occurrence of epithelial lifting than control fish or those exposed to 0.03 mg L^{-1} UAN, but were not significantly different from fish exposed to other concentrations of reagent grade or metabolic ammonia. Data relating to hypertrophy, lamellar fusion and aneurysm were heterogeneous following transformation, evidence of a high degree of variability between replicates.

Epitheliocystis was observed in control fish and fish exposed to all ammonia concentrations (see Appendix A [Frances et al., 1997] for a full description of epitheliocystis in silver perch). Cysts were observed in 96% of samples and from all but one aquarium. The condition affected 2-96% of gill filaments, with a prevalence of up to 75%. Those fish exposed to 0.02 mg L^{-1} UAN of metabolic origin had a significantly ($P<0.05$) lower occurrence of epitheliocystis than control fish. No significant difference in prevalence of epitheliocystis was observed between control fish and those exposed to any concentration of reagent ammonia.

Histopathological material was examined and photographed as described in Chapter 2 - General Materials and Methods. Plate 5 illustrates epithelial lifting in gill tissue of fish exposed to 0.02 mg L^{-1} UAN (metabolic). Although all histopathological data were heterogeneous other than that for epithelial lifting, illustrations are provided of lamellar fusion (Plate 6 - gill tissue of fish exposed to 0.07 mg L^{-1} UAN - note the presence of epitheliocystis), aneurysm (Plate 7 - gill tissue of fish exposed to 0.14 mg

TABLE 13

Percentage of filaments showing histopathological changes for silver perch exposed to sub-lethal concentrations of ammonia for 39 days.

UAN (mg L^{-1})	% of Filaments Affected[1,2,3]				
	Epithelial lifting	Epithelio-cystis	Hypertrophy of epithelial cells[4]	Lamellar fusion[4]	Aneurysm[4]
0.01	10.2±2.3[a]	9.3±1.6[a]	3.3±0.9	6.9±1.1	0.3±0.3
0.02[5]	12.3±1.6[ab]	3.2±1.3[b]	3.1±0.7	3.5±0.7	0.2±0.1
0.03	8.0±1.4[a]	7.6±1.5[ab]	4.2±0.8	6.6±1.5	0.2±0.1
0.04	12.3±1.4[ab]	4.1±1.4[ab]	2.9±0.8	3.2±0.8	0.1±0.1
0.07	10.5±1.7[ab]	8.4±1.9[ab]	3.5±1.0	3.4±0.9	0.0±0.0
0.14	13.4±1.7[ab]	6.0±1.6[ab]	3.2±1.0	4.1±1.1	0.1±0.1
0.36	16.9±1.9[b]	6.1±1.8[ab]	3.6±1.0	6.3±1.1	0.1±0.1

1 Values are means ± s.e.

2 Within columns, different letters in the superscript indicate a significant difference ($P<0.05$)

3 Data were subjected to arcsine transformation prior to analysis

4 Data were heterogeneous following transformation

5 Metabolic ammonia

Plate 5 Epithelial lifting in fish exposed to 0.02 mg L^{-1} UAN of metabolic origin for 39 days (x 100 magnification).

Plate 6 Lamellar fusion in fish exposed to 0.07 mg L^{-1} UAN for 39 days. Epitheliocystis (e) is also present (x 100 magnification).

Plate 7 Aneurysm in fish exposed to 0.14 mg L^{-1} UAN for 39 days (x 400 magnification).

Plate 8 Extensive lamellar fusion and epitheliocystis (e) in fish exposed to 0.36 mg L^{-1} UAN for 39 days (x 100 magnification).

100um

Plate 5

100um

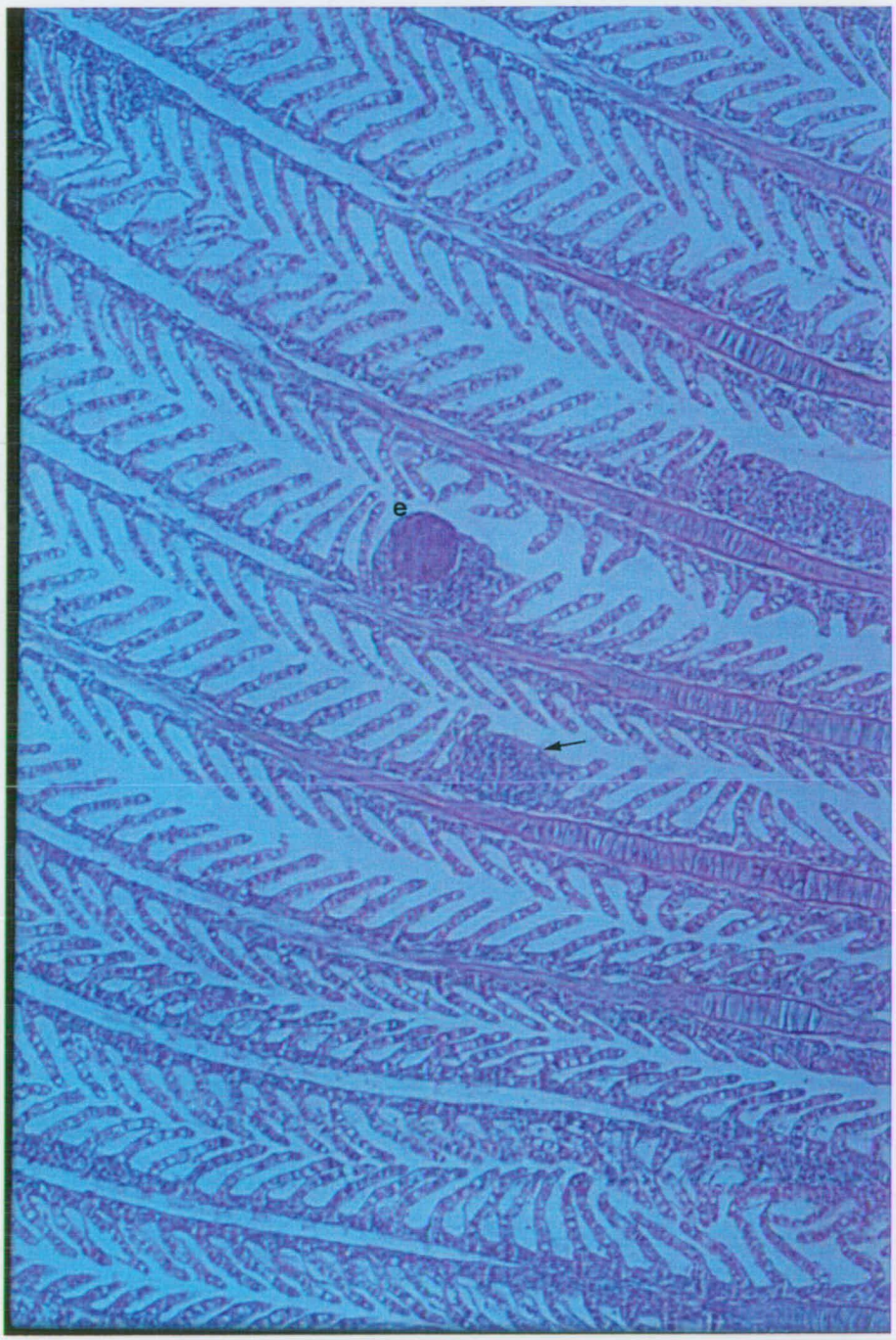

Plate 6

100um

Plate 7

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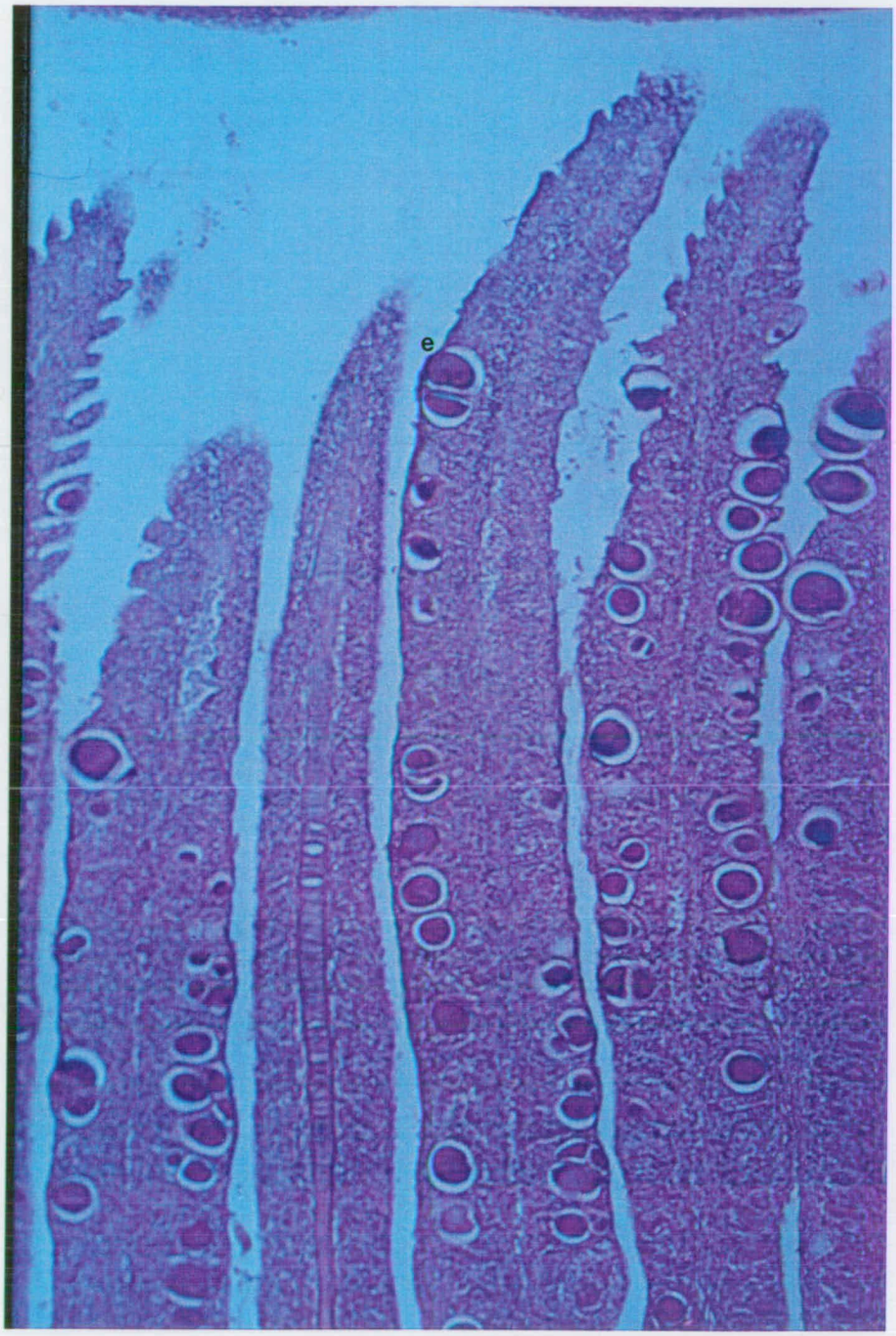

Plate 8

L^{-1} UAN), and extensive lamellar fusion accompanied by epitheliocystis (Plate 8 - gill tissue of fish exposed to 0.36 mg L^{-1} UAN).

Discussion

Overall survival was greater than 99%, and was not affected by exposure to up to 0.36 mg L^{-1} UAN for 39 days. The environmental conditions (other than ammonia concentration) established in this experiment were designed to optimise the growth of silver perch. Growth was significantly reduced following exposure to 0.36 mg L^{-1} UAN. Using linear regression, the concentration at which growth is estimated to be reduced by 5% is 0.06 mg L^{-1} UAN. The sensitivity of silver perch to ammonia intoxication is similar to other cultured temperate species. The growth of 7 day old channel catfish *I. punctatus* was significantly affected by exposure to 0.212 mg L^{-1} UAN (Bader and Grizzle, 1992), while Colt and Tchobanoglous (1978) showed that growth of juveniles of this species was affected by a 31 day exposure to 0.31 mg L^{-1} UAN. However, in work with juveniles of the same species, Robinette (1973) observed no growth when fish were exposed to 0.12 mg L^{-1} UAN for 27 days. Mallett and Sims (1994) showed a significant reduction in growth of carp *Cyprinus carpio* fry exposed to 0.35 mg L^{-1} UAN for 31 days and roach *Rutilus rutilus* fry exposed to 0.31 mg L^{-1} UAN for 130 days.

Juvenile silver perch appear to be more sensitive to ammonia toxicity than are tilapia *Tilapia aurea.* Redner and Stickney (1979) observed no mortalities in juvenile tilapia *T. aurea* exposed to 0.43-0.53 mg L^{-1} UAN for 35 days. Conversely, silver perch are less sensitive than rainbow trout *O. mykiss* to ammonia. The growth of

rainbow trout *O. mykiss* fry was significantly affected by exposure to 0.13 mg L^{-1} UAN for 12 months (Smith and Piper, 1975). Similarly, Soderberg et al. (1983) found that growth of this species was correlated with the average daily maximum UAN concentration in ponds, and that concentrations above 0.12 mg L^{-1} UAN affected growth.

Specific growth rate (SGR) was higher for control fish than for fish exposed to any concentration of ammonia, although the difference was significant only at 0.36 mg L^{-1} UAN (Table 12). Fish in the present experiment showed better SGR values than similar fish used in nutrition growth experiments (G. L. Allan, unpublished data) or the control fish in the nitrite growth-limiting experiment reported in this thesis. In addition, there was a significant difference in feed consumption between control fish and those exposed to 0.07 mg L^{-1} UAN, but the difference in feed consumption between control fish and fish exposed to higher ammonia concentrations (0.14 and 0.36 mg L^{-1} UAN) was not significant. Food Conversion Ratio (FCR) was not significantly different for any of the concentrations tested. Since growth of fish exposed to 0.36 mg L^{-1} UAN was significantly depressed while food consumption was not significantly different, this indicates that some of the energy derived from the relatively high feed consumption of fish exposed to 0.36 mg L^{-1} UAN may have been expended on metabolic maintenance rather than growth. Shuter (1990) suggested that more energy may be required for metabolic maintenance in a sub-optimal or stressful environment. Furthermore, Jeney et al. (1992a) found that exposure to ammonia elicited a primary stress response in common carp *C. carpio*. Therefore, silver perch in the present study may have responded to the stressful environment of the high concentration ammonia treatment by expending more energy

on metabolic maintenance, such that, even though consumption was not reduced in this group, growth was negatively impacted.

Published literature indicates that susceptibility to ammonia toxicity may be affected by life stage and/or age in rainbow trout *O. mykiss* (Rice and Stokes, 1975; Thurston and Russo, 1983) but not in channel catfish *I. punctatus* (Bader and Grizzle, 1992) or fathead minnows *Pimephales promelas* (Thurston et al., 1983). It is yet to be established if differences in susceptibility to ammonia intoxication exist between life stages of silver perch, and hence the results of this study can only, with confidence, be applied to silver perch of a similar size.

Exposure to 0.36 mg L^{-1} UAN significantly increased the percentage of filaments affected by epithelial lifting (Table 13). Epithelial lifting results in an increased diffusion distance across the membrane, reducing the gills' functional surface area (Peters et al., 1984) and capacity for efficient gas exchange (Smart, 1976). Such a condition may be benign in well oxygenated water, but when subjected to conditions of low dissolved oxygen the functional impairment of respiration may result in growth retardation (Larmoyeux and Piper, 1973) or death (Downing and Merkens, 1955; Merkens and Downing, 1957). Following a six month exposure, the concentration of UAN at which gill lesions (including fusion and hyperplasia of gill epithelium, hypertrophy of epithelial cells and telangiectasis) occur in rainbow trout *O. mykiss* was estimated to be 0.013-0.022 mg L^{-1} (Thurston et al., 1984). This supports the earlier finding of comparative resistance of silver perch to ammonia toxicity relative to rainbow trout *O. mykiss* (Chapter 3 of this thesis).

The attempt to discriminate histopathological differences between reagent and metabolic ammonia in the present study is confounded by elevated metabolic nitrite concentrations in the metabolic ammonia aquaria (Table 11). As discussed elsewhere in this thesis (Chapter 6 - The Short-Term Growth-Limiting Concentration of Nitrite for Silver Perch), a reagent nitrite concentration of 1.1 mg L^{-1} NO_2-N significantly increased the incidence of epithelial lifting in juvenile silver perch. It is not known whether a difference in morphological response exists between metabolic and reagent nitrite, although a conservative interpretation suggests that a concentration of 1.1 mg L^{-1} NO_2-N of metabolic origin would elicit a similar histopathological response to exposure to 1.1 mg L^{-1} NO_2-N of reagent origin. As the mean metabolic nitrite concentration in the metabolic ammonia aquaria was 1.91 mg L^{-1} NO_2-N, the effects of elevated metabolic ammonia and of elevated metabolic nitrite cannot be separated. Both metabolic ammonia and metabolic nitrite may be the causative agents of epithelial lifting in the metabolic ammonia aquaria in the present study. Ammonia and nitrite may act synergistically or antagonistically (Meade, 1985; Bader and Grizzle, 1992). In addition, other growth inhibiting factors produced as metabolites and accumulated under crowded conditions, such as urea and uric acid, amine and amine oxide derivatives, creatine and creatinine, may contribute to functional impairment and growth suppression (Brockway, 1950; Daoust and Ferguson, 1984; Meade, 1985). In the present study, exposure to 0.36 mg L^{-1} UAN ammonia significantly increased the occurrence of epithelial lifting, but made no significant difference to the occurrence of other observable histopathology. It is therefore concluded that ammonia intoxication in the present study had little effect on gill histopathology.

Epitheliocystis observed in this experiment, although severe, occurred as a benign infection and had no apparent effect on survival or growth rate. Silver perch exposed to metabolic ammonia had a significantly ($P<0.05$) lower percentage of gill filaments affected by cysts than did controls, but the difference between fish exposed to reagent ammonia and metabolic ammonia was not significant ($P>0.05$). This suggests that the prevalence of epitheliocystis is neither closely correlated with the source of ammonia nor with its concentration, and implies that epitheliocystis was a pre-condition of the juvenile fish used in this experiment. However, subsequent histopathological examination of cohort silver perch retained at NSW Fisheries' Grafton Research Centre showed no evidence of epitheliocystis (J. Frances, unpublished data). This somewhat supports the conjecture that exposure to ammonia encouraged the development of an otherwise benign and difficult-to-detect condition, but fails to explain the occurrence of epitheliocystis in control fish. Perhaps stress attributable to transport conditions between Grafton Research Centre and Port Stephens Research Centre (approximately 8 hours by road) contributed to the outbreak of epitheliocystis observed in this study.

Growth was not negatively impacted by the presence of epitheliocystis in this study, as indicated by a high growth rate relative to nutrition experiments conducted using similar facilities (G. L. Allan, unpublished data). Epitheliocystis has been shown to cause benign infection in cultured freshwater species, including fingerling channel catfish *I. punctatus* (Zimmer et al., 1984), chronic infection with associated 0.4% day^{-1} mortality in juvenile carp *C. carpio* (Voronin and Chernysheva, 1997), while it has been associated with massive mortalities in juvenile yellowtail *Seriola mazatlana* during nursery culture (Venizelos and Benetti, 1996). Although no deleterious effect

of infection is evident in the present study, it remains possible that under different culture conditions, or with fish of a different life-stage, gill function, growth and/or survival may be affected.

The important conclusion of this study is that the ammonia concentration beyond which growth of juvenile silver perch is reduced by 5% is 0.06 mg L^{-1} UAN. It is recommended that ammonia levels be measured regularly during pond production of silver perch and be maintained below this threshold concentration to maximise production.

The incidence of epithelial lifting may be a useful histopathological indicator of growth reducing ammonia intoxication in silver perch. It is recommended that histological preparations be undertaken on gills of fish during routine health monitoring to examine the incidence of epithelial lifting.

An adjunct of this study was the discovery, for the first time, of epitheliocystis in silver perch. Although the infection in this instance was benign, numerous published studies indicate that epitheliocystis may cause mass mortalities in hatcheries. It is therefore recommended that silver perch be monitored regularly (ie broodstock prior to transfer into hatcheries, and fry/fingerlings prior to dispatch from hatcheries), using standard histological techniques, for the presence of cysts, as described in Frances et al. (1997; Appendix 1), which are indicative of epitheliocystis infection.

CHAPTER 6

The Short-Term Growth-Limiting Concentration of Nitrite for Silver Perch

Introduction

The chronic toxicity of nitrite to freshwater fish varies considerably (see reviews of Lewis and Morris [1986], Russo and Thurston [1991], and Russo [1992]). The differences between published results can be attributed in part to differences in susceptibility between species (Palachek and Tomasso, 1984a). In addition, differences in other water quality variables (especially chloride [Perrone and Meade, 1977], pH [Wedermeyer and Yasutake, 1978; Russo et al., 1981], dissolved oxygen [Watenpaugh and Beitinger, 1986], and calcium [Crawford and Allen, 1977; Tomasso et al., 1980]) at experimental facilities is a confounding factor.

Perrone and Meade (1977) suggested gill damage at extreme environmental concentrations may be the mechanism of nitrite toxicity in coho salmon *Oncorhynchus kisutch*. Michael et al. (1987) found epithelial lifting and necrosis, and hyperplasia and hypertrophy of epithelial cells of gills of juvenile African catfish *Clarius lazera* exposed to 3.2 mg L^{-1} NO_2-N for 6 months. Wedermeyer and Yasutake (1978) also observed hypertrophic lamellar epithelium and swelling of epithelial cells in steelhead trout *Oncorhynchus mykiss* gill lamellae following 6 months exposure to 0.06 mg L^{-1} NO_2-N. However, Colt et al. (1981) observed no histological damage in channel catfish *Ictalurus punctatus* exposed to 4.8 mg L^{-1}

NO_2-N for 31 days. Thus there is some conjecture in the literature regarding the relationship between gill histopathology and nitrite toxicity in freshwater fish.

The objective of this study was to determine the concentration of nitrite which impacts on the short-term growth of juvenile silver perch. Furthermore, histological techniques were used to investigate if structural changes in gill tissue are induced by exposure to nitrite.

Methods

Fish

Fish were sourced as described in General Materials and Methods. Prior to stocking the experiment, fish were graded to exclude fish outside the weight range 6-7 g. The fish were lightly anaesthetised with benzocaine (*p* aminobenzoate at 20 mg L^{-1}) and individually weighed, and each aquarium was stocked using random interspersion with 7 fish (6.49±0.03g; x±se; n=126). The fish were allowed to acclimate to experimental conditions for 7 days, and were fed as described in Chapter 2 - General Materials and Methods. Two fish died during acclimation and were replaced with fish of a similar size.

Survival, growth and gill histopathology were the variables used to evaluate the effect of the toxicant.

Experimental Procedures

Concentrated Analytical Reagent grade sodium nitrite (Na NO_2) solutions were prepared using laboratory test water as diluent and placed into 15 L reservoirs. Further experimental procedures are as detailed in Chapter 2 - General Materials and Methods. Eighteen 70L aquaria were established for this experiment; 3 replicates of each nitrite concentration and of the control. Test concentrations were assigned to each aquarium via a randomisation procedure. Concentrations tested were 0.02, 1.1, 1.9, 4.0, 8.0 and 16.2 mg L^{-1} NO_2-N.

Throughout acclimation and the experiment, fish and aquaria were maintained as described in Chapter 2 - General Materials and Methods.

Dead fish were removed from aquaria and weighed, and all fish were individually weighed at the end of the experiment. Mortalities were replaced with fin-clipped fish of a similar size to maintain stocking density, with feed rates adjusted accordingly. No mortality occurred in any of the control aquaria. The experiment was terminated after 25 days exposure to toxicant.

Temperature and dissolved oxygen were maintained at 24.8-26.4°C and 6.2-8.4 mg L^{-1} respectively, and the pH varied from 7.9 to 8.4. Photoperiod was maintained at 12 h light:12 h dark.

Water Quality

Nitrite concentrations were determined daily according to the method described in Chapter 2 - General Materials and Methods. Ammonia concentrations were determined weekly for all aquaria using the method described in Chapter 2 - General Materials and Methods. Dissolved oxygen, temperature, salinity and pH were measured daily using Yeokal Water Quality Meter Model 611, as described in Chapter 2 - General Materials and Methods. Alkalinity and hardness were determined at the completion of the experiment using commercial test kits (Aquasonics Pty Ltd, Ingleburn, NSW, Australia). Colourimetric measurements were determined as described in Chapter 2 - General Materials and Methods. Values for these variables are presented in Table 14.

Histology

Five fish per aquarium (90 fish in total) were killed using benzocaine *p* aminobenzoate overdose at 50 mg L^{-1} and sampled for histological preparations. Gill tissue was prepared for histological examination as described in Chapter 2 - General Materials and Methods. At least twenty intact filaments per sample were observed and histopathological changes recorded as percent of filaments affected. Fin-clipped replacement fish were excluded from histological examination.

TABLE 14

Water quality data during the nitrite growth-limiting experiment.

	Temp	DO	pH	Ammonia		Salinity	Alkalinity	Hardness
				TAN	UAN			
	°C	mg L^{-1}		mg L^{-1}	mg L^{-1}	(‰)	(mg L^{-1})	(as Ca CO_3)
mean	25.9	6.7	8.2	0.08	0.007	0.4	90	75
se	0.015	0.032	0.004	0.004	0.0003	0.003	-	-
max	26.4	8.4	8.4	0.16	0.014	0.6	-	-
min	24.8	6.2	7.9	0.03	0.003	0.3	-	-

Statistical Analysis

Statistical analysis was undertaken as described in Chapter 2 - General Materials and Methods. A two-phase linear regression model (Sedgwick, 1979) was used to estimate the EC_0 and EC_5 concentrations (the concentration causing no reduction in growth, and that at which growth is reduced by 5%, respectively). The mean growth of the fish in the control aquaria and in those treatments which did not significantly reduce growth was used to establish a horizontal line for the first phase. The second phase included data from the highest concentration where growth was not significantly reduced compared with the controls and data from all other concentrations, and was linearly regressed. The intersection of the horizontal line and the linear regression was used as an estimate of the concentration at which growth was affected (EC_0). The EC_5 value was calculated from the linear regression.

Results

Survival

Overall survival was high, with less than 4% mortality. Single mortalities were recorded from each of four aquaria; two replicates from each of 4.0 and 8.0 mg L^{-1} NO_2-N respectively. Fin-clipped replacement fish survived for the remaining course of the experiment.

Growth

Silver perch exposed to increasing concentrations within the range tested exhibited reduced growth. Nitrite (NO_2-N mg L^{-1}) concentration significantly effected ($P<0.05$) wet weight gain, specific growth rate (SGR) and food conversion ratio (FCR; Table 15).

Control fish showed a significantly ($P<0.05$) better (higher) SGR than those fish exposed to 4.0 and 16.2 mg L^{-1} NO_2-N. Feed consumption between treatments did not differ significantly, however FCR was significantly ($P<0.05$) higher (poorer) at a concentration of 16.2 mg L^{-1} NO_2-N (Table 15). The concentration beyond which growth is reduced is 1.43 mg L^{-1} NO_2-N (Fig 4). The EC_5 is 2.78 mg L^{-1} NO_2-N.

Histology

No gross pathology of gill tissue was observed. A comparison of histopathological changes to gill filaments following nitrite exposure shows little evidence of significant trends (Table 16). Data relating to percentage of filaments affected by lamellar fusion, aneurysm and hyperplasia were heterogeneous, indicating a high degree of variability between replicates. There was a significant difference ($P<0.05$) in the percent of filaments affected by epithelial lifting and hypertrophy (due to the swelling of cells) between control treatment and those exposed to 1.1 mg L^{-1} NO_2-N. In the case of epithelial lifting, control fish showed a significantly lower percentage of filaments affected than did fish exposed to 1.1 mg L^{-1} NO_2-N. With respect to hypertrophy, control fish exhibited a significantly higher percentage of filaments

TABLE 15

Wet weight gain, specific growth rate (SGR), food conversion ratio (FCR) and food consumption of silver perch exposed to sub-lethal concentrations of nitrite.

NO_2-N (mg L^{-1}) Nitrite	Wet weight gain (g $fish^{-1}$)[1,2]	SGR[1,2]	FCR[1,2]	Food consumption (g)[1,2]
0.02±0.00	3.4±0.5^{a}	1.7±0.2^{a}	1.8±0.1^{a}	5.5±0.6^{a}
1.1 ±0.02	2.9±0.1^{ab}	1.5±0.0^{ab}	1.9±0.2^{a}	6.0±0.7^{a}
1.9 ±0.04	2.9±0.4^{ab}	1.5±0.1^{ab}	2.1±0.2^{a}	4.5±0.3^{a}
4.0 ±0.09	2.8±0.1^{ab}	1.4±0.0^{ab}	2.1±0.1^{a}	4.6±0.4^{a}
8.0 ±0.16	2.2±0.1^{b}	1.1±0.0^{b}	1.7±0.1^{a}	5.1±0.6^{a}
16.2 ±0.21	1.4±0.2^{c}	0.8±0.1^{c}	3.3±0.3^{b}	5.8±0.2^{a}

1 Values are means ± s.e.

2 Within columns, different letters in the superscript indicate a significant difference ($P<0.05$).

SGR Specific Growth Rate (% day^{-1}): $\frac{(\ln \text{final wt} - \ln \text{initial wt})}{\text{time (days)}} \times 100$

FCR Food Conversion Ratio: $\frac{\text{Feed fed}}{\text{wet weight gain}}$

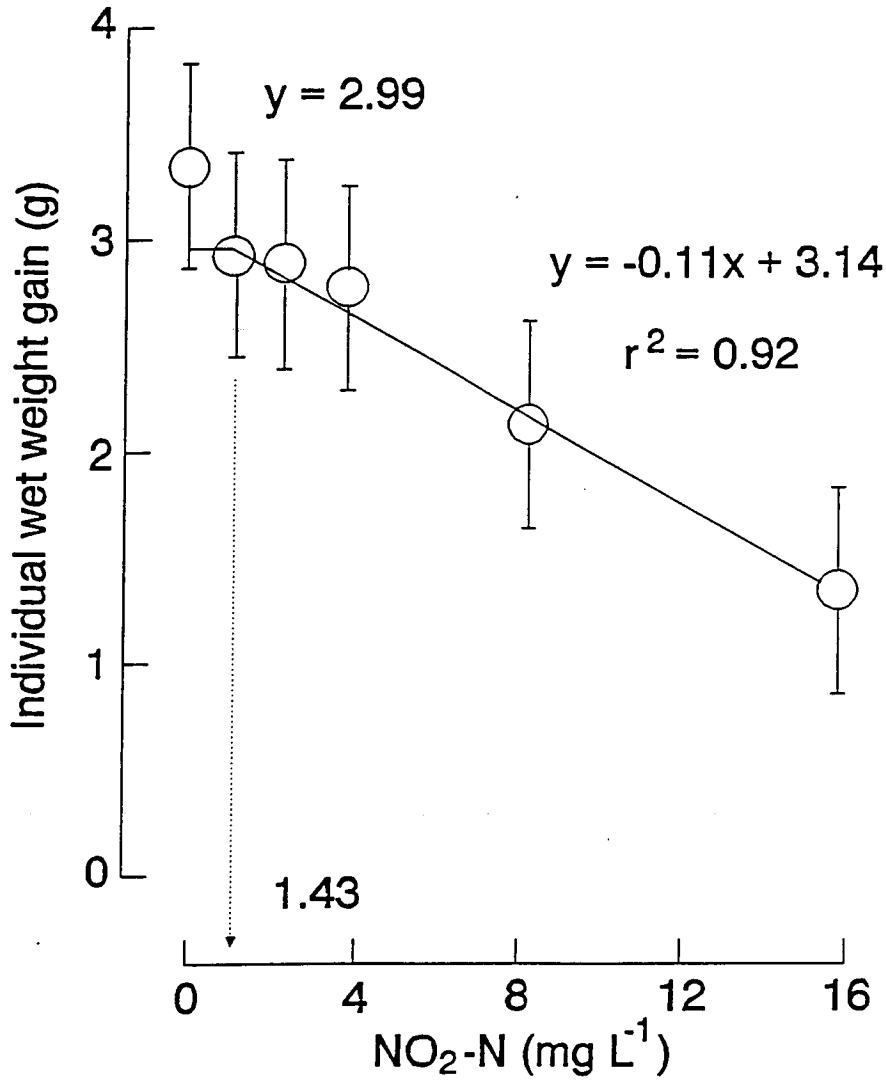

Figure 4 Regression analysis of individual wet weight gain for juvenile silver perch exposed to nitrite

TABLE 16

Percentage of gill filaments showing histopathological changes for silver perch exposed to sub-lethal concentrations of nitrite for 25 days.

NO_2-N (mg L^{-1})	Filaments Affected (%) [1,2,3]				
	Epithelial lifting	Hypertrophy	Lamellar fusion[4]	Hyperplasia[4]	Aneurysm[4]
0.02	6.1±2.6[a]	10.0±1.7[a]	0.0±0.0	0.5±0.5	0.5±0.3
1.1	22.1±3.5[b]	3.2±1.0[b]	0.0±0.0	0.5±0.3	0.6±0.4
1.9	13.7±4.3[ab]	8.9±1.7[ab]	0.3±0.3	0.3±0.3	0.3±0.3
4.0	8.1±2.2[ab]	9.4±1.8[ab]	0.6±0.4	0.0±0.0	0.0±0.0
8.0	17.7±4.0[ab]	4.5±1.3[ab]	0.0±0.0	2.4±1.7	0.0±0.0
16.2	17.9±4.9[ab]	5.1±1.7[ab]	0.7±0.5	2.7±1.5	1.0±0.7

1 Values are means ± se

2 Within columns, different letters in the superscript indicate a significant difference ($P<0.05$).

3 Data were subjected to arcsine transformation prior to analysis.

4 Data were heterogeneous following transformation.

affected than did fish exposed to 1.1 mg L^{-1} NO_2-N. However, there was no concentration related increase in percentage of filaments affected by any other lesion (Table 16).

Histopathological material was examined and photographed as described in Chapter 2 - General Materials and Methods. Plate 9 illustrates gill tissue from fish exposed to 1.1 mg L^{-1} NO_2-N, demonstrating epithelial lifting. Plate 10 illustrates aneurysm observed in gill tissue of fish fish exposed to 1.9 mg L^{-1} NO_2-N.

Discussion

Survival of silver perch was not affected by exposure to up to 16.2 mg L^{-1} NO_2-N for 25 days. There is a paucity of published literature with which to compare this result, however, in their study of juvenile channel catfish *I. punctatus*, Colt et al. (1981) reported a significant increase in mortality at a nitrite concentration of 3.71 mg L^{-1} NO_2-N, while mortality of European eel *Anguilla anguilla* was high and variable in all treatments, including the controls (Kamstra et al., 1996).

Although survival was not affected within the range tested, the growth of silver perch was significantly affected by exposure to nitrite at concentrations above 1.43 mg L^{-1} NO_2-N. The EC_5 for juvenile silver perch is 2.78 mg L^{-1} NO_2-N. Previously reported growth reduction in juvenile channel catfish *I. punctatus* occurred at 1.62 mg L^{-1} NO_2-N (Colt et al., 1981), indicating a similar sensitivity to nitrite exposure for silver perch and channel catfish *I. punctatus*. In comparison, Wedermeyer and Yatsutake (1978) found no significant growth reduction in juvenile steelhead trout

Plate 9 Epithelial lifting in fish exposed to 1.1 mg L^{-1} NO_2-N for 25 days (x 400 magnification).

Plate 10 Aneurysm in fish exposed to 1.1 mg L^{-1} NO_2-N for 25 days (x 400 magnification).

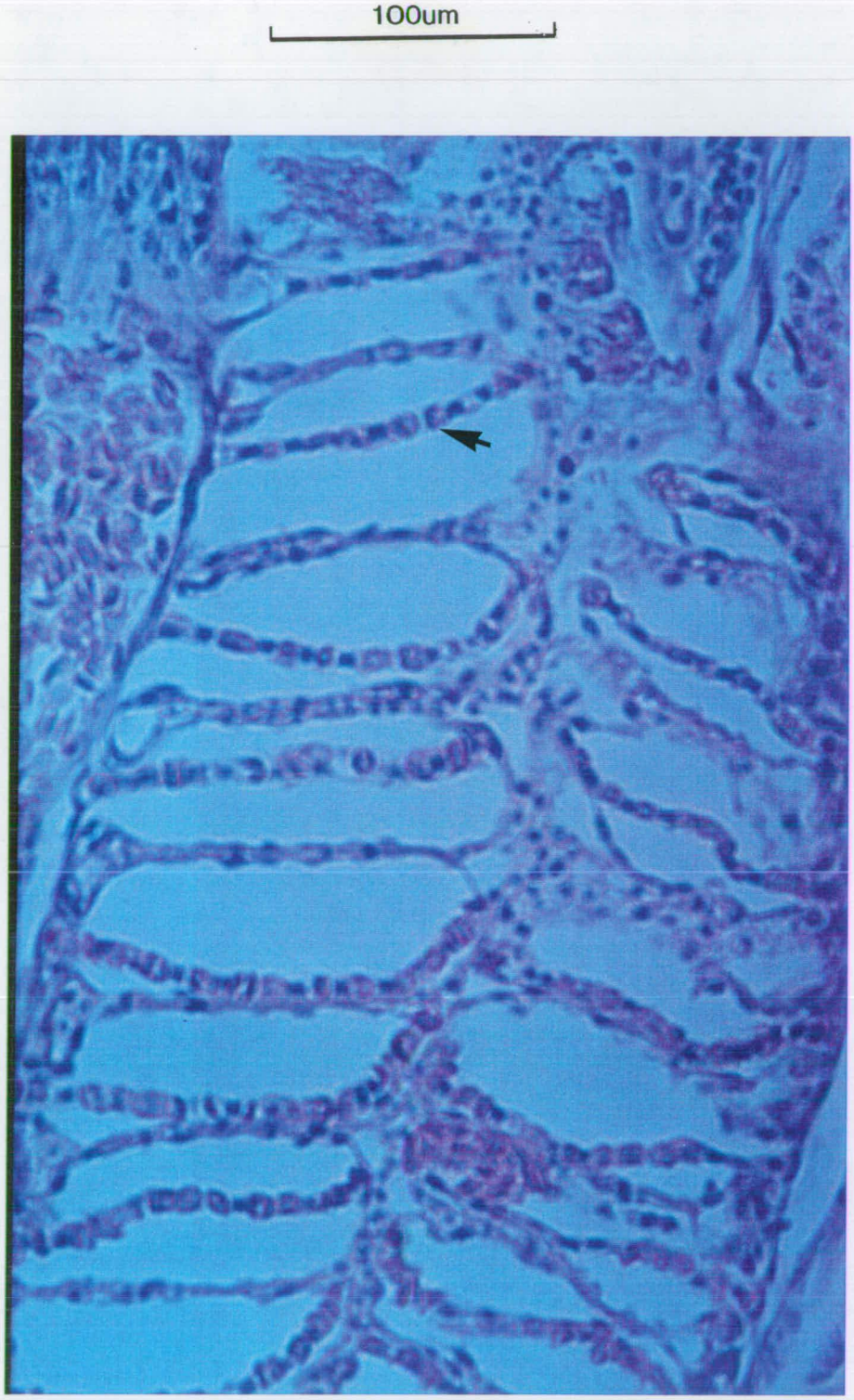

Plate 9

100um

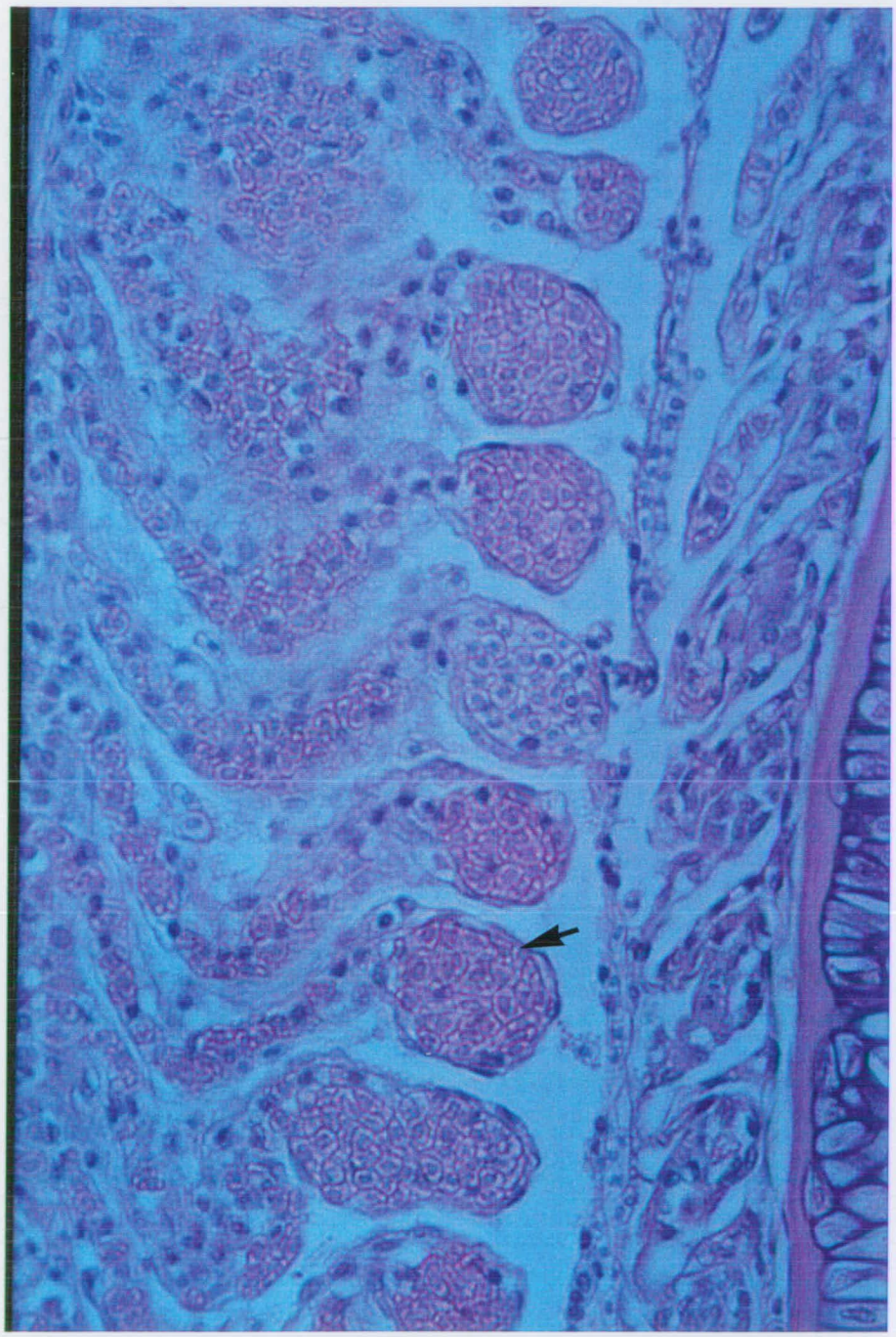

Plate 10

O. mykiss exposed to 0.03 mg L^{-1} NO_2-N for 6 months. More recently, Kamstra et al. (1996) found no significant effect of nitrite on growth of European eel *A. anguilla* exposed to 20 mg L^{-1} NO_2-N for 50 days, showing that European eels are less sensitive to chronic nitrite exposure than either silver perch or channel catfish.

Fingerlings exposed to 16.2 mg L^{-1} NO_2-N had a significantly lower Specific Growth Rate (SGR) than those exposed to lower concentrations ($P<0.05$; Table 15). SGR for control fish was higher than for fish exposed to any concentration of nitrite, though the difference was significant only for fish exposed to 4.0 mg L^{-1} NO_2-N ($P<0.05$). No significant difference in feed consumption was found (Table 15), indicating that exposure to nitrite did not suppress appetite. There was no apparent relationship between feed consumption and differences in SGR in the present study, therefore the poorer SGR observed for fingerlings exposed to 8.0 and 16.2 mg L^{-1} NO_2-N may be due to an increase in energy required for metabolic maintenance in a sub-optimal or stressful environment, as suggested for other species (Shuter, 1990). More energy is expended on maintenance in relation to growth, and hence similar food intake will result in poorer growth when fish are subjected to toxicant-induced stress. Control fish in the present experiment showed similar SGR values to silver perch controls used in nutrition growth experiments (G. L. Allan, unpublished data).

Silver perch fingerlings exposed to 16.2 mg L^{-1} NO_2-N had a significantly poorer FCR than fish exposed to lower concentrations of nitrite and controls ($P<0.05$; Table 15). This is consistent with this study's findings with respect to SGR and may also result from increased energy expenditure on maintenance relative to growth under elevated nitrite conditions. In contrast to the present study, Kamstra et al. (1996)

found no significant effect of exposure to elevated nitrite concentrations for 50 days on growth, SGR or FCR in European eels *A. anguilla*. However, high mortality was observed in all treatments, including the controls, and as mortalities were apparently not replaced, this may have resulted in a density-related effect between treatments which would make interpretation of growth data more difficult (Sprague, 1985).

The present study revealed a significant difference in the histology of the gills of silver perch exposed to 1.1 mg L^{-1} NO_2-N for 25 days compared with control fish (Table 16). The percentage of filaments affected by epithelial lifting was not significantly different for exposures above 1.1 mg L^{-1} NO_2-N, while the percentage of filaments affected by hypertrophy was significantly ($P<0.05$) higher in controls than in those fish exposed to 1.1 mg L^{-1} NO_2-N only. It therefore appears that histological change may be highly variable and indicate no discernible pattern of effect of nitrite concentration on gills. As exposure to higher concentrations of nitrite revealed no significant increase in percentage of filaments affected by any of the histopathological features observed, it may be concluded that although histopathological changes were observed in gill tissue of fish exposed to sub-acute concentrations of nitrite relative to control fish, these changes were neither dose-dependent nor consistent among fish within a treatment.

Wedemeyer and Yasutake (1978) observed no changes in gill histology of juvenile steelhead trout *O. mykiss* exposed to 0.03 mg L^{-1} NO_2-N for 6 months. This exposure level appears low and may have been below that necessary to detect an effect on gill histology. However, Michael et al. (1987) found hypertrophy and hyperplasia consistently occurred in juvenile African catfish *C. lazera* and some evidence of

epithelial lifting and epithelial cell necrosis when exposed to 2.8 mg L^{-1} NO_2-N for 6 months. Lightner et al. (1988) also observed gill hyperplasia in cultured tilapia *Oreochromis* sp., but could not differentiate if this was attributable to chronic ammonia or chronic nitrite toxicity.

Background water quality variables have been shown to affect the toxicity of nitrite to freshwater fish (Crawford and Allen, 1977; Wedermeyer and Yasutake, 1978). Many studies have established the protective effect of chloride on nitrite toxicity for a variety of species (Crawford and Allen, 1977; Perrone and Meade, 1977; Almendras, 1987; Bowser et al., 1983; Eddy et al., 1983; Mazik et al., 1991; Weirich et al., 1993). In the present study, salinity was maintained at 0.3 - 0.6‰, and the results presented herein can be expected to be observed in silver perch subjected to similar water quality parameters. Further studies exploring the effect of chloride on nitrite toxicity to silver perch are recommended.

A well documented effect of nitrite is its ability to convert haemoglobin to methemoglobin, a form unable to carry oxygen (Brown and McLeay, 1975; Wedermeyer and Yasutake, 1978). Under high nitrite concentrations, the reduction of haemoglobin to methemoglobin has been observed to cause the blood and gills to brown and darken (Smith and Williams, 1974; Smith and Russo, 1975; Huey et al., 1980; Scarano et al., 1984), and is therefore commonly referred to as "brown blood disease" (Konikoff, 1975) or methemoglobinaemia. Silver perch in the present study exhibited no abnormal gross gill pathology. However, although no macroscopically observable sign of methemoglobinaemia was noted in this experiment, it is possible that some conversion of haemoglobin to methemoglobin took place in the blood of

fish in this study. Methemoglobinaemia has been observed in silver perch exposed to higher concentrations of nitrite (see Chapter 4 in this thesis). The nitrite concentrations to which fish were exposed in the short-term growth-limiting experiment within the present study were comparative to those which have elicited methemoglobinaemia in published studies on other species (Bowser et al., 1983; Almendras, 1987). The absence of macroscopically observable browning of blood and gill tissues in the present study indicates that silver perch may possess a mechanism by which protection from nitrite intoxication is inferred, such as the nitrite exclusion mechanism found in largemouth bass *Micropterus samoides* (Palachek and Tomasso, 1984a).

Results from the present study indicate that silver perch possess moderate sensitivity to the sub-lethal effects of nitrite toxicity. Exposure of juvenile silver perch to nitrite concentrations above 1.43 mg L^{-1} NO_2-N reduces growth. Data from the present experiment indicate that growth is more closely correlated to nitrite toxicity than is gill histopathology in juvenile silver perch. The potential of a protective mechanism by which resistance to nitrite toxicity is enhanced in silver perch is suggested, and requires further investigation.

CHAPTER 7

General Discussion

The present study focused on the effects of ammonia and nitrite on the juvenile life stage of silver perch. Juvenile fish, relative to their adult counterparts, exhibit fast growth and possess the ability for a high level of food consumption (Martinez-Tapia and Fernandez-Patio, 1991). Aquaculture typically relies on rearing juveniles at relatively high densities. In many culture systems, the products of protein catabolism, such as ammonia, nitrite and suspended solids, can accumulate, especially in static systems or those with restricted water exchange (Colt and Armstrong, 1981; Person Le-Ruyet et al., 1995). In general, larvae are more susceptible to ammonia toxicity than adults (Meade, 1985), however hatcheries are usually provided with sufficient control of water quality such that ammonia does not reach toxic concentrations, and fingerlings (<0.5-2.0 g) are typically raised in fertilised ponds with no, or minimal, feed inputs. Thus, this study addressed the most sensitive life stage which would potentially be exposed to toxic concentrations of ammonia and nitrite. The results of this study will assist in the management of grow-out facilities for silver perch.

The results of this study refer to juvenile silver perch. Life stage has been demonstrated to affect susceptibility to toxicants in some species. Burkhalter and Kaya (1977) found rainbow trout *Oncorhynchus mykiss* eggs were more resistant to ammonia intoxication than fry. Likewise, Rice and Stokes (1975) and Solbe and Shurben (1989) also found a negative correlation between age and susceptibility to

ammonia intoxication in their comparative study of fry and adults of the same species. Mallett and Sims (1994), in their study of carp *Cyprinus carpio* and roach *Rutilus rutilus*, found newly hatched larvae and fry of both species were more sensitive to ammonia toxicity than their eggs, but that this sensitivity decreased with fry age. However, Bader and Grizzle (1992) found no age-related differences in susceptibility to UAN for 1 day old larvae and 7 day old juvenile channel catfish *Ictalurus punctatus*. The results of the present study relate to juvenile silver perch and cannot be applied to other life stages of this species with confidence.

Differences in susceptibility of different life stages to nitrite intoxication have also been established in the literature. Studies by Russo et al. (1974) and Russo and Thurston (1977) found adult rainbow trout *O. mykiss* to be slightly more sensitive to nitrite than juveniles and larvae. Further, Perrone and Meade (1977) found coho salmon *Oncorhynchus kisutch* fry to be more resistant to nitrite toxicity than yearlings. Yearlings exhibited elevated methemoglobin levels and mortality when exposed to nitrite, while fry showed neither. Almendras (1987) stated that juvenile milkfish *Chanos chanos* have a greater tolerance to nitrite than their adult counterparts and suggested they may have a more efficient methemoglobin reductase system. It is not known if differential resistance to nitrite exists for silver perch of different life stages. Further work in this area would be useful to aquaculturists. However, this study focussed on the critical age.

Size may also influence the susceptibility of freshwater fish to toxicants. Thurston et al. (1981c) found that rainbow trout *O. mykiss* larger that 2 kg were more vulnerable to acute exposure to ammonia than fish in the size range 20-300 g. However,

Thurston et al. (1983) found no effect of fish size on ammonia toxicity for fathead minnows *Pimephales promales* ranging from 0.1 to 2.3 g. The present study was conducted using juvenile silver perch in the size range 1.8-3.6 g, with similar average weights (2.36 g cf 1.99 g). Therefore, this study establishes that silver perch of this size are moderately resistant to both acute and growth-limiting concentrations of ammonia, and perform similarly to other temperate freshwater species. Further studies are necessary to determine if life stage or size-dependent susceptibility exists for this species.

Furthermore, there is some discrepancy in the literature regarding the effect of fish size on nitrite toxicity. In their comprehensive review, Lewis and Morris (1986) stated that small fish are generally more resistant to nitrite than larger fish of the same species, and cited published studies on coho salmon *O. kisutch* (Perrone and Meade, 1977) rainbow trout *O. mykiss* (Smith and Williams, 1974) and fathead minnows *P. promales* (Palachek and Tomasso, 1984b) in support of their argument. However, Wedermeyer and Yasutake (1978) found 5 g steelhead trout *O. mykiss* to be more sensitive to acute nitrite toxicity than 10 g fish. In their study of African catfish *Clarias lazera.* Hilmy et al. (1987) found 65 g fish to be slightly more sensitive to acute nitrite toxicity than 166 g fish. In contrast, Russo (1980) found no significant difference in susceptibility to nitrite toxicity of rainbow trout *O. mykiss* in the size range 2 to 387g. It is stressed that, given the above variability in published results, the results from the present study can only be applied to fish of a similar size to those used in the nitrite experiments herein (4.2 to 8.8 g). However, as the average weight of fish in the present study was similar (6.7 g cf 6.49 g), it is reasonable to assume negligible differences in susceptibility between fish from different nitrite

experiments herein. As size is therefore discounted as contributing to the resistance to acute nitrite intoxication observed in this study, the argument for the possession of a nitrite exclusion mechanism which imparts resistance at relatively high nitrite concentrations is further supported. How useful the results of the present study are in terms of predicting toxic responses of silver perch of different sizes and/or life stages is yet to be established. Further toxicity studies on larger silver perch would be useful.

In the wild, silver perch is classified as vulnerable (Schiller et al., 1997). This has clear implications for the silver perch aquaculture industry, particularly in terms of maintaining sufficient genetic diversity of broodstock, and thereby of their offspring. It is yet to be established whether genetic differences which affect susceptibility to toxicants exists between silver perch broodstock sourced from different areas. Published studies of other aquaculture candidates (Axiak, 1991) indicate that such differences are possible.

Due to logistical constraints, the acute and the two growth-limiting experiments in the present study were conducted using different cohorts of silver perch, which may have possessed different resistance to the toxicants investigated. Variability between batches of fish is typical (Person Le-Ruyet et al., 1995), and a number of factors contribute to this variability, including size, life stage, history, genetic differences, food quality and intake and the interaction of the toxicant under investigation with other water quality variables (Meade, 1985; Axiak, 1991; Russo and Thurston, 1991). A comparison of growth and food consumption data (SGR and FCR) for the control fish in the two growth-limiting experiments in the present study indicates

that, although the initial size of fish was similar (1.8-2.2 g [ammonia experiment] compared with 6-7 g [nitrite experiment]), the control fish in the ammonia experiment grew faster (better SGR) but used food less efficiently (poorer FCR) than control fish in the nitrite experiment. The slight difference in initial weight (0.4 g) may have contributed to observed differences. As these fish were of different cohorts, genetic differences and/or history also may have contributed to this variability.

Different cohorts of silver perch may have had different opportunities to develop resistance to ammonia and nitrite through acclimation in rearing ponds or holding facilities. Tucker and Schwedler (1983) found acclimation over 5-7 days inferred resistance to nitrite toxicity in channel catfish *I. punctatus,* and suggested that this may be due to improved efficiency of the haemoglobin reductase system. In their study of the same species, Urrutia and Tomasso (1987) observed an ability to acclimate to nitrite, but that acclimation is concentration- and time-dependent. Alabaster et al. (1979) observed an increase in median 24h LC_{50} for ammonia of at least 38% in salmon smolts *Salmo salar* acclimated for 1 day to a concentration of ammonia approaching the 24 h LC_{50}. Every effort was undertaken in the present study to minimise variability of water quality conditions in the holding facilities, with background UAN concentrations never exceeding 0.01 mg L^{-1} UAN and background nitrite concentrations never exceeding 0.02 mg L^{-1} NO_2-N. However, the conditions to which the silver perch used in this study were subjected to as fry were possibly more variable, due to water supply constraints (S. J. Rowland, pers. comm.).

Furthermore, exposure to fluctuating concentrations of ammonia has been shown to influence susceptibility. Thurston et al. (1981c) exposed rainbow trout *O. mykiss* and cutthroat trout *Salmo clarki* to fluctuating ammonia concentrations and found this exposure conferred some resistance to acute ammonia toxicity. Fish were more tolerant of constant than fluctuating concentrations in that study. Larmoyeux and Piper (1973) showed rainbow trout *O. mykiss* exposed to high UAN, accompanied by low DO, developed a tolerance to those conditions, while un-acclimated fish did not. However, Soderberg (1985) established a stronger correlation between histopathological changes and daily maximum, rather than daily average, ammonia concentration for the same species. The present study was conducted so as to achieve consistent exposure to a given concentration of toxicant. However, water quality conditions to which silver perch are exposed under farming conditions are likely to fluctuate. The effect of exposure to fluctuating concentrations of ammonia and nitrite on susceptibility of silver perch would be useful information to aquaculturists. If exposure to fluctuating concentrations of toxicant conferred some resistance to ammonia intoxication in silver perch, as established in the studies above, then the results of the present study would be an under-estimation of the resistance of silver perch to ammonia and nitrite toxicity.

Both ammonia and nitrite have been shown to act as stressors on freshwater fish (Tomasso, 1994). The release of cortisol is a primary stress response in fish (Oppenborn and Goudie, 1993). Elevated levels of ammonia have been shown to give rise to the release of corticosteroids into blood plasma of channel catfish *I. punctatus* (Tomasso et al., 1981) and rainbow trout *O. mykiss* (Swift, 1981), while elevated levels of adrenalin have been reported in carp *C. carpio* exposed to

ammonia (Jeney et al., 1992a). Similarly, nitrite has been demonstrated to cause an elevation in corticosteroids in striped bass *Morone saxatilis* (Mazik et al., 1991).

Exposure of fish to a toxicant may induce a stress response: in addition fish in a stressed state may succumb more readily to secondary infections (Plumb, 1984; Sprague, 1985). Dysfunction of the immune system gives rise to a reduction in disease resistance in a variety of species of freshwater fish (Wedermeyer, 1970; Adams, 1990). Ammonia intoxication has been identified as a predisposing factor in the occurrence of bacterial gill disease in a number of species (Burrows, 1964; Larmoyeux and Piper, 1973; Schreckenbach et al., 1975; Hanson and Grizzle, 1985; Tarazona et al., 1987). In addition, Thurston et al. (1984) and Ferguson (1988) noted an association between elevated ammonia levels and an increase in protein droplets in urinary tubular epithelium and nephrosis in rainbow trout *O. mykiss*. Similarly, Lightner et al. (1988) observed skin and gill protozoan parasitism, bacterial septicaemia and fungal infections in a variety of tilapia species, including *Oreochromis mossambicus*, *O. aureus*, *O. mossambicus* x *Tilapia zillii* and *O. mossambicus* x *O. urolepis hornorum* following exposure to elevated levels of both ammonia and nitrite. Plumb (1984) also observed that channel catfish *I. punctatus* exposed to a combination of elevated ammonia and reduced dissolved oxygen were susceptible to bacterial infection, while Burkhalter and Kaya (1977) reported blue-sac disease in rainbow trout *O. mykiss* fry exposed to ammonia.

In the present study, silver perch in the chronic ammonia exposure experiment exhibited a high incidence of epitheliocystis, including those fish in the control treatment which were exposed to only background ammonia concentrations (see

Appendix 1 for a full description). The incidence of epitheliocystis in the present study was benign, and no correlation was established between exposure to ammonia and the severity of epitheliocystis outbreak. However, epitheliocystis has been shown to cause mass mortalities in other cultured freshwater species, including steelhead trout *O. mykiss* (Rourke et al., 1984) and lake trout *Salvelinus namaycush* (Bradley et al., 1988). It is possible that mortalities resulting from epitheliocystis may have been precluded by the termination of the present study after 39 days. Furthermore, longer term studies may allow the development of other, less benign, diseases.

Health status also affects disease resistance in fish (Schaperclaus et al., 1992). Possible differences in the general health status of the two cohorts of fish used in the present study, due to a variety of factors, may have contributed to differences in susceptibility to stressors. The higher glycogen storage levels observed in the livers of coho salmon *O. kisutch* in good physical condition may allow for the provision of energy requirements via anaerobic glycolysis, reducing reliance on DO and thereby indirectly conferring resistance to nitrite toxicosis (Perrone and Meade, 1977). Downing and Merkens (1955) and Alabaster et al. (1979) also established a positive correlation between susceptibility to ammonia and decreasing concentrations of DO in salmonids. Hence differences in health status of the two cohorts of silver perch used in the present study may have affected their resistance to both or either of the toxicants investigated.

The quality of the diet fed to fish influences their health and disease resistance (Halver, 1989; Lovell, 1989) Throughout the present study, silver perch were fed a

35% protein ration (Allan and Rowland, 1992). However, the quality and availability of Vitamin C in the diets fed to the two silver perch cohorts in the present study may have varied. Vitamin C is highly heat-labile (Lovell, 1989), and therefore transport and storage of diets can greatly affect their nutritional quality. Dietary Vitamin C has been shown to inhibit nitrite-induced methemoglobinaemia in channel catfish *I. punctatus* (Wise et al., 1988). In addition, the same species fed a Vitamin C-free diet showed an increased susceptibility to ammonia intoxication (Mazik et al., 1987).

In the 96 h ammonia experiment in the present study, pH was adjusted to 8, with variation from 7.8 to 8.2. The highest degree of pH variability was recorded for those fish exposed to the highest concentration of UAN (see Chapter 3 in this thesis), due to the depressing effect of the addition of NH_4Cl on pH. In the case of the growth-limiting experiment, pH was maintained within the range 7.9-8.2, once again with the highest degree of variability recorded for those fish exposed to the highest concentration of UAN. The effect of subtle pH variations on ammonia toxicity has not been established for silver perch, and evidence from published studies varies such that a trend cannot be anticipated. Tomasso et al. (1980) found the 24 h LC_{50} of UAN for channel catfish *I. punctatus* at pH 8 to be significantly higher than at pH 7 or 9. Sousa et al. (1974) found the resistance of chinook salmon *Oncorhynchus tshawytscha* smolts to ammonia toxicity was increased by mildly acidic culture conditions. However, Thurston et al. (1981a) observed an increase in the acute toxicity of UAN to rainbow trout *O. mykiss* and fathead minnows *P. promelas* at pH 6.5 compared with pH 9. Furthermore, following 32 days' exposure to ammonia, Broderius et al. (1985) reported a similar result for smallmouth bass *Micropterus*

dolomieui at pH 6.6 compared with 8.7. The optimal pH for silver perch has not been established, although the recommended pH range (6.5 to 9.0; Rowland, 1995b) for pond culture indicate that silver perch are tolerant of a wide range of pH conditions. It is not known if slight variations in pH at the pH ranges observed in the present study are sufficient to affect the toxicity of UAN for silver perch. Total ammonia was maintained at constant levels throughout both experiments, therefore if differences in degree of toxicity were experienced, it was probably due to UAN fluctuations. An understanding of the influence of pH on the toxicity of ammonia to silver perch would be useful to aquaculturists.

The range of pH in the 96 h nitrite experiment of the present study was between 7.8 and 8.3, and in the nitrite growth-limiting experiment was between 7.85 and 8.14. pH influences the nitrite equilibrium and therefore the relative proportions of nitrite and nitrous acid. Eddy et al. (1983) suggested that gill membranes of salmonids may be impermeable to NO_2, but permeable to HNO_2, which dissociates in the blood according to pH. Meade and Perrone (1980) concurred, and suggested that nitrite toxicity may be, in part, nitrous acid toxicity. Colt and Tchobanoglous (1976), in their study of acute nitrite toxicity on channel catfish *I. punctatus* established linear mortality curves for nitrite and nitrous acid and found their data better fit the nitrous acid curve. Russo et al. (1981) calculated 96 h LC_{50} values for NO_2-N and HNO_2-N toxicity to rainbow trout *O. mykiss* and found that as pH increases the toxicity of nitrite decreases, while that of nitrous acid increases. Wedermeyer and Yasutake (1978) found that increasing pH from 6 to 8 decreased nitrite toxicity substantially. This is consistent with the nitrous acid toxicity hypothesis. However, Huey et al. (1982) found enhanced nitrite toxicity in bluegill *Lepomis macrochirus* at high pH,

and attributed toxicity at low pH to nitrous acid, which they suggested passes rapidly across the gills, giving rise to profound methemoglobinaemia. They further suggested that the slower passage of nitrite across the gills at high pH allows the functioning of the methemoglobin reductase system before lethal concentrations are reached. Colt et al. (1981) also suggested that nitrous acid may be the toxic form of nitrite, and stated that in this context the major effect of the addition of Cl^- or Ca^{2+} would be the resultant increased ionic strength which would act on the NO_2-HNO_2 equilibrium and reduce the proportion of nitrous acid present. However, Huey et al. (1982) in their study of the combined effects of pH and chloride concentrations on nitrite toxicity to bluegill *L. macrochirus* found no decrease in nitrite toxicity at low pH (4.0) and high chloride concentrations. It should be noted, however, that in that study there may have been an interaction between chloride, nitrite and phosphate buffers. Given that the pH ranges in both nitrite experiments in the present study fluctuated by less than 1 pH unit, the relative proportions of nitrous acid and nitrite varied slightly. However, in this pH range, less than 1% nitrous acid exists (Tomasso, 1994), and nitrous acid is therefore unlikely to have influenced toxicity in the present study.

The 96 h ammonia experiment in the present study was conducted at 25.7-26.4°C. Likewise, temperature in the growth-limiting experiment in the present study was maintained at 24.7-27.2°C. Such conditions are considered to be near-optimal for silver perch (Rowland, 1995b). Temperature also affects the position of the ammonia dissociation equilibrium, with increasing temperature favouring an increasing proportion of total ammonia present as UAN (Emerson et al., 1975; Ferguson, 1988). A 10°C increase in temperature doubles the concentration of UAN

present in an ammonia solution (EIFAC, 1970). Ministry of Technology (1968) found a threefold increase in the 24 h LC_{50} for rainbow trout *Oncorhynchus mykiss* with an increase in temperature from 5 to 18°C. However, more recent studies report ammonia to be less toxic at temperatures approaching optimal for a given species (Colt and Tchobanoglous, 1976; Thurston and Russo, 1983). Thurston et al. (1983) found the acute toxicity of UAN to fathead minnows *P. promelas* decreased as temperature increased between 12 and 22°C. Further, Rosenboom and Richey (1977) report similar trends for UAN toxicity to bluegill *L. macrochirus*, channel catfish *I. punctatus* and largemouth bass *M. salmoides*. In addition, Bader and Grizzle (1992) found the toxicity of UAN to channel catfish *I. punctatus* was reduced with higher temperatures and pHs. As the present study was conducted at temperatures considered to be near-optimal for silver perch, it appears reasonable to deduce that 1.2 mg L^{-1} UAN is close to the maximum 96 h tolerance for silver perch at any temperature, while 0.06 mg L^{-1} UAN approaches the concentration at which growth of juvenile silver perch is reduced by 5%.

Elevated ammonia levels have been shown to impair the oxygen carrying capacity of the blood (Brockway, 1950), and several studies have established an inverse relationship between ammonia toxicity and DO for salmonids. Alabaster et al. (1979) found the toxicity of UAN to Atlantic salmon *S. salar* smolts was increased at low DO, and Downing and Merkens (1955) found the susceptibility of rainbow trout *O. mykiss* to UAN decreased as DO was raised from 1.5 to 8.5 mg L^{-1}. In work with the same species, Lloyd (1961) showed ammonia toxicity to be elevated by decreasing DO and suggested this may result from the increased respiratory rate and hence increased volume of water passing over the gills. In studies of rainbow trout

O. mykiss, perch *Perca fluviatilis*, roach *R. rutilus* and gudgeon *Gobio gobio*, Merkens and Downing (1957) found decreasing DO to increase the toxicity of UAN to all species studied, with the exception of gudgeon, which showed no significant difference in susceptibility. Thurston et al. (1981b) found UAN toxicity to rainbow trout *O. mykiss* increased as DO decreased from 8.6 to 2.6 mg L^{-1} and was more strongly correlated for short-term exposures. These authors estimated the UAN tolerance at 5 mg L^{-1} DO to be 30% less than at 8.5 mg L^{-1} DO. However, Thurston et al. (1983) found no significant relationship between DO and ammonia toxicity to fathead minnows *P. promelas* between 3 and 9 mg L^{-1} DO. Dissolved oxygen concentrations in the 96 h experiment in present study were maintained at 6.4 to 7.2 mg L^{-1}, representing 80% and 90% saturation, respectively, and 5.4 to 6.3 mg L^{-1} , representing 70% and 80% saturation, respectively, in the growth limiting experiment. Dissolved oxygen was therefore unlikely to impact on ammonia toxicity in the present study.

Exposure to nitrite is known to impair haemoglobin's ability to reversibly bind with oxygen (Bowser et al., 1983). As a result, haemoglobin is converted to methemoglobin, otherwise referred to as ferrihaemoglobin (Kiese, 1974). Methemoglobinaemia is said to compromise a fish's ability to tolerate low dissolved oxygen conditions (Bowser et al., 1983; Watenpaugh and Beitinger, 1986). In addition, nitrite exposure may impart a reduced capacity for sustained activity (Huey et al., 1984), due to its effects on the blood's oxygen carrying capacity. Dissolved oxygen concentrations were maintained at a moderate level (Chapters 4 and 6, respectively) throughout both the 96 h and the growth-limiting experiments in this

study, therefore reducing the impact of the possible presence of methemoglobinaemia on inducing anoxia.

A number of adverse effects have been associated with exposure of freshwater fish to ammonia. These include histopathological damage to the gills (Smart, 1976; Colt and Armstrong, 1981), neurological dysfunction (Daoust and Ferguson, 1984) and paralysis (Lumsden et al., 1993). One possible explanation for the number and variety of hypotheses regarding toxic mechanism is that there may be a difference in the mechanism by which toxicity is conferred which is concentration- and/or exposure-dependent. However, Meade (1985) noted that the accumulated results of published studies indicated that UAN concentration was probably not the prime agent in inducing gill hyperplasia. In addition, given the evidence of the possession of an active transport mechanism in some species (Evans and Cameron, 1986), differences in toxic mechanism almost certainly exist between species, which may further complicate interpretation of histopathology. Results of the 96 h experiment and the growth-limiting experiment in the present study indicate a significant increase in the percentage of filaments affected by epithelial lifting following exposure to elevated concentrations of UAN. Epithelial lifting increases the diffusion distance across the respiratory epithelium (Mallatt, 1985), which may impair the fish's ability to respire in poorly oxygenated waters (Larmoyeux and Piper, 1973). This study establishes epithelial lifting as a useful indicator of both acute ammonia intoxication and of growth reducing concentrations of ammonia and recommends the routine use histopathology in silver perch aquaculture management. It is stressed that epithelial lifting can be induced as an artefact of poor fixation, and attention to correct histological procedures is fundamental to the useful application of

histopathology as an indicator of ammonia intoxication. However, there is insufficient evidence to establish the primary mechanism of acute ammonia toxicity in silver perch. Neurological dysfunction is suspected and further studies are recommended.

The attempt to discriminate histopathological differences between Analytical Reagent and metabolic ammonia was confounded by elevated levels of nitrite in the growth-limiting experiment. In this case, the effects of chronic ammonia intoxication and chronic nitrite intoxication could not be discriminated. An inability to discern between the effects of ammonia and nitrite was also experienced by Lightner et al. (1988), in their study of various tilapia species.

Results of the 96 h nitrite experiment in the present study revealed no difference in histopathological changes with exposure to increasing concentrations of nitrite. Similarly, results of growth-limiting experiment in the present study indicate a high degree of variability in histological changes to gill tissue which were neither dose-dependent nor consistent within a treatment. In contrast, Perrone and Meade (1977) suggested gill damage at extreme environmental concentrations may be the mechanism of nitrite toxicity in coho salmon *O. kisutch*. Lightner et al. (1988) also observed gill hyperplasia in cultured tilapia *Oreochromis* sp., but could not differentiate if this was attributable to chronic ammonia or chronic nitrite toxicity. Michael et al. (1987) found hypertrophy and hyperplasia consistently occurred in juvenile African catfish *C. lazera,* and some evidence of epithelial lifting and epithelial cell necrosis when exposed to 2.8 mg L^{-1} NO_2-N for 6 months. Wedemeyer and Yasutake (1978) observed no changes in gill histology of juvenile steelhead trout

O. mykiss exposed to 0.03 mg L^{-1} NO_2-N for 6 months. This exposure level appears low and may have been below that necessary to detect an effect on gill histology. However, Colt et al. (1981) observed no histological gill damage in channel catfish *I. punctatus* exposed to 1.6 mg L^{-1} NO_2-N for 31 days. The present study indicates that gill histopathology was not a useful indicator of physiological impairment associated with loss of ability to orientate (96 h experiment) or reduced growth (growth-limiting experiment) of juvenile silver perch following exposure to nitrite. The results of the growth-limiting experiment in the present study suggest the possession of a protective mechanism which infers resistance to chronic nitrite intoxication. Further research in this area is recommended.

The proportion of UAN present in a given ammonia solution decreases with increasing salinities (Alabaster et al., 1979; Bower and Bidwell, 1978). On examination of the data of Herbert and Shurben (1965), Alabaster and Lloyd (1980) suggested that the trend of decreasing toxicity of ammonia to rainbow trout *O. mykiss* up to 30% seawater then increasing toxicity up to 100% seawater reflects the concentration isotonic with fish blood. Results of experiments by Harader and Allen (1983) on chinook salmon *O. tshawytscha* concurred, with 9.6‰ salinity inferring a greater degree of resistance to ammonia toxicity than higher or lower salinities tested. Similarly, Sousa et al. (1974) found intermediate salinities to be beneficial regarding ammonia toxicity to the same species and suggested this may be due to an exchange mechanism between sodium and ammonium ions across the gills. Thurston et al. (1979) suggested that, in general, the toxicity of ammonia at higher salinities is not due to the NH_4^+/NH_3 ratio alone and must be influenced by some other mechanism(s). The present study was conducted at 0‰. However, silver perch have

been shown to tolerate salinities up to 12‰ (Guo et al., 1995), and saline baths are an established disease management tool (Rowland and Ingram, 1991). Based on the other published studies mentioned above, the evidence suggesting protection from ammonia toxicity through increasing salinity, at least to isotonicity, is strong. Further investigation of the effect of salinity on ammonia toxicity to silver perch would be useful.

In their comprehensive review, Lewis and Morris (1986) stressed the importance of reporting chloride concentrations in nitrite toxicity studies, as without such data meaningful comparisons between studies cannot be made. The presence of chloride ions reduces the toxic effect of nitrite in salmonids (Crawford and Allen, 1977; Meade and Perrone, 1980; Eddy et al., 1983), in milkfish *C. chanos* (Almendras, 1987), in channel catfish *I. punctatus* (Bowser et al., 1983), in striped bass *M. saxatilis* (Mazik et al., 1991), and in sunshine bass *Morone chrysops* x *M. saxatilis* (Weirich et al., 1993). Salinities which provided nitrite toxicity protection in these studies ranged from 16 to 32.5‰. Furthermore, Mitchell and Cech (1983) observed a confounding effect of residual chlorine on ammonia-induced gill damage to channel catfish *I. punctatus* and stressed the importance of using contaminant-free water in aquatic toxicology studies. Both experiments in the present study were conducted at 0-0.6‰. In addition, the present study was conducted using sand- and cartridge-filtered bore water, which is not affected by chlorine. The effect of NaCl on nitrite toxicity is yet to be established for silver perch, however, the absence of chloride ions in the present study precludes any contribution from chloride ions towards the observed resistance of silver perch to nitrite toxicity. Furthermore, Lewis and Morris (1986) stated that the protective effect of chloride is greatest for

the most sensitive species, and least for the most resistant. If this is also true for silver perch, we can expect the effect of chloride on the toxicity of nitrite to silver perch to be relatively minor for chronic exposure. The effect of chloride on acute nitrite toxicity may have useful applications for the aquaculture industry.

The background levels of cations, particularly calcium, in freshwater is often high (Lewis and Morris, 1986) and varies greatly with water source. Tomasso et al. (1980) implicated environmental calcium in decreasing ammonia toxicity to channel catfish *I. punctatus*, perhaps by decreasing gill membrane permeability, allowing the efflux of elevated internal ammonia levels. Ferguson (1988) concurred, stating that elevated calcium levels increase the ammonia tolerance of fish, either by decreasing gill permeability or by forming carbonic acid and thereby lowering the pH at the gill epithelium. Source water and hence background water quality variables are likely to vary widely between laboratories and also between commercial aquaculture facilities. The results of published studies on other freshwater species suggest that background water quality parameters are likely to affect the susceptibility of silver perch to toxic insult, and hence application of the results of the present study should be undertaken with respect to the background water quality variables presented in Chapters 3, 4, 5 and 6 in this thesis.

Cations have also been reported to influence the toxicity of nitrite. Provision of protection from nitrite toxicity by calcium chloride has been established for a number of species, including milkfish *C. chanos* (Almendras, 1987), steelhead trout *O. mykiss* (Wedemeyer and Yasutake, 1978), striped bass *M. saxatilis* (Mazik et al., 1991) and sunshine bass *M. chrysops* x *M. saxatilis* (Weirich et al., 1993). Tomasso

and Carmichael (1991) suggested that calcium may inhibit the diffusion of nitrite by altering membrane permeability. Furthermore, Crawford and Allen (1977) found the addition of calcium sulphate to freshwater reduced the toxicity of nitrite to chinook salmon *O. tshawytscha*. In addition, Wedemeyer and Yasutake (1978) found a large reduction in the acute toxicity of nitrite to steelhead trout *O. mykiss* when total water hardness ($CaCO_3$) was increased from 25 to 3000 mg L^{-1}. Source water used in the present study was filtered bore water, with a calcium content of 65-75 mg L^{-1} (Chapters 3, 4, 5 and 6 of this thesis). The above findings demonstrate the necessity of reporting alkalinity and hardness of source water to allow for meaningful comparisons with other published studies. The influence of calcium on nitrite toxicity may be species-specific. As the impact of other water quality have been shown to be synergistic, the impact of this and other water quality variables may be dependent on the presence, form and concentration of a suite of other water quality variables. The effect of different background water quality variables on the toxicity of ammonia and nitrite to silver perch would be useful to aquaculturists.

In general, the 96 h LC_{50} for most species studied has been found to be 5 to 10 times the growth reducing concentration (Wedermeyer and Yasutake, 1978; Colt et al., 1981). Lewis and Morris (1986) stated that there is no evidence of detrimental effects to freshwater fish from nitrite concentrations less than 10% of the 96 h LC_{50}. The results of the present study do not support this, as the estimated 96 h LC_{50} was approximately 160 mg L^{-1} NO_2-N , while the concentration above which growth was reduced is 1.43 mg L^{-1} NO_2-N, a factor of approximately 100. It is therefore possible that silver perch possess a different mechanism of susceptibility to nitrite toxicity than other freshwater fish, and that though they are highly resistant to acutely toxic

concentrations of nitrite, comparatively low concentrations reduce growth. Colt et al. (1981) suggested that different sites of action for lethal and sublethal effects of nitrite on channel catfish *I. punctatus* may exist. This may be the case for silver perch also.

The results of the present study suggest that silver perch may possess a mechanism by which protection from nitrite intoxication is inferred. Published studies to date indicate some evidence for a nitrite exclusion mechanism in some species. Palachek and Tomasso (1984a) found that largemouth bass *M. salmoides* do not concentrate nitrite in plasma at higher concentrations to that of the environment and suggested that this species is better able to discriminate between Cl^- and NO_2^- ions at the cell membrane. Furthermore, the relationship between percent methemoglobin and environmental nitrite is not always linear (Brown and McLeay, 1975; Smith and Williams, 1974), indicating the possible involvement of another mechanism in conferring toxicity. A methemoglobin reductase system has been described in white spotted char *Salvelinus leucomaunis* by Miyauchi et al. (1979) and in channel catfish *I. punctatus* by Huey and Beitinger (1982). This mechanism enables the reversal of methemoglobin back to haemoglobin within 24-48 h of the fish's return to nitrite-free water (Almendras, 1987). The presence of a methemoglobin reductase system may confound analysis of blood chemistry and interpretation of such results. It is yet to be established if silver perch possess such a mechanism. Further studies in this area would be useful.

It appears likely that there is a relationship between salinity tolerance, the number of chloride cells in the gills, the possession of a nitrite exclusion mechanism and susceptibility to nitrite toxicity. Silver perch is a native of the inland waters of

eastern Australia, a predominantly freshwater environment, where saline ground waters may influence river salinity at times. Guo et al. (1995) found that juvenile silver perch tolerated direct transfer from fresh water to 12‰ salinity without mortality. Tolerance of elevated salinity is an established tool for disease management in freshwater fish aquaculture (Rowland and Ingram, 1991) and may in addition confer a degree of resistance to nitrite toxicity, as has been found with other freshwater fish. Salinity in the present study at no time exceeded 0.6‰ (Chapters 3, 4, 5, and 6 in this thesis). Further studies on the effect of salinity and nitrite exposure on blood chemistry and gill histopathology are required to determine if chloride ions provide silver perch with increased resistance to nitrite toxicity.

In conclusion, the present study has highlighted a number of areas where further research would be useful. These include the investigation of:

i) a nitrite protective mechanism, haematology, methemoglobin production and blood chemistry, particularly with respect to chronic exposure to nitrite;

ii) a suspected relationship between acute ammonia toxicity and neurological dysfunction;

iii) the mode of action of acute nitrite intoxication and other organs, eg brain, kidney and liver;

iv) the relationship between different life stage, size and genetics on susceptibility to ammonia and nitrite;

v) the effect of exposure to fluctuating concentrations of ammonia and nitrite on susceptibility;

vi) the effect of different source water (especially with respect to hardness) on ammonia and nitrite intoxication;

vii) the influence of subtle pH variations, particularly at levels close to optimal, on ammonia toxicity;and

viii) the influence of chloride ions on acute nitrite toxicity.

The present study concludes that for juvenile silver perch, the estimated 96 h LC_{50} for ammonia is 1.2 mg L^{-1} UAN and for nitrite is 160 mg L^{-1} NO_2-N. The growth of juvenile silver perch is reduced at 0.36 mg L^{-1} UAN and 1.43 mg L^{-1} NO_2-N. The disparity between the concentrations of nitrite which result in acute and chronic toxicity suggests that different mechanisms and/or organs may be involved in susceptibility to short and long term exposures. The resistance shown by silver perch to acute nitrite toxicity is suggestive of the possession of a nitrite exclusion mechanism.

Epithelial lifting is an indicator of ammonia intoxication in juvenile silver perch, and routine histopathology would therefore be a useful management tool in terms of ammonia toxicity for silver perch aquaculturists. However, no relationship was established between gill histopathology and nitrite toxicity. The discovery during this study of epitheliocystis in silver perch further encourages the routine use of histology in silver perch culture.

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Appendix

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It has been published as: Frances, J., Tennent, R., Nowak, B. F. 1997, Epitheliocystis in silver perch, Bidyanus bidyanus (Mitchell), Journal of fish diseases, 20(6), 453-457.